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INHIBITION OF APOPTOSIS AND TRANSCRIPTION FACTOR BINDING ACTIVITY BY ZINC PYRITHIONE IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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A thesis submitted to the School of Graduate Studies at the University of Ottawa in Partial Fulfillment of the Requirements for the Degree of Master of Science in Physiology

Supervisor: Dr. Henry Fliss

ABSTRACT

Cell death has been characterized as occurring by one of two ways: necrosis or apoptosis. Necrotic cell death is passive, resulting from severe trauma, whereas apoptotic death requires the active participation of the cell and occurs in response to a signal that triggers a characteristic series of events. The actual mechanisms regulating the apoptotic pathway have yet to be fully elucidated. However, regulation appears to depend on the balance between apoptosis-suppressing and apoptosis-inducing factors within the cell. Accordingly, apoptosis may be initiated by the activation or suppression of transcription factors involved in the regulation of apoptotic factors. The ability of zinc to inhibit apoptosis has been well documented, but the mechanisms of this protection have yet to be elucidated. In view of the ability of zinc to alter transcriptional events, we sought to determine if zinc may prevent apoptosis by altering transcription factor binding activity. Of particular interest were the transcription factors Nuclear Factor kappa B (NFκB), Activator Protein-1 (AP-1), and Sp1, which have previously been shown to be associated with apoptosis.

The initial studies correlated changes in transcription factor binding activity with the induction of apoptosis. Human umbilical vein endothelial cells (HUVEC) were exposed to ionizing radiation (IR) or were treated with N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), a zinc chelator, or tumour necrosis factor α (TNFα) and were assessed for apoptosis following 8 hours of treatment. Apoptosis was significantly increased in IR and TPEN treated cells when compared to control levels, as assessed by histological examination of Hoechst 33258 stained nuclei. TNFα did not induce apoptosis above control levels. Apoptosis was confirmed using DNA gel electrophoresis, which displayed prominent DNA 'laddering', a hallmark of apoptosis. The binding activity of NFκB, AP-1 or Sp1 was determined using electrophoretic mobility shift assays. Nuclear extracts from cells treated for only two hours were incubated with DNA fragments containing consensus sequences to these transcription factors, and were subjected to electrophoresis. The changes in transcription factor binding activity were then compared with

the susceptibility to apoptosis. Our data show that while both IR and TPEN induced significant apoptosis in HUVEC, only IR caused a significant increase in NFκB binding activity over control levels. TPEN-treated cells exhibited significantly lower Sp1 binding activity. TNFα did not induce apoptosis, although it did potently activate NFκB. The increases in nuclear NFκB binding activity induced by IR and TNFα were associated with corresponding decreases in the level of the cytosolic inhibitor, IκB, as assessed by Western blot. The activity of AP-1 remained unchanged by the above treatments.

Subsequent studies sought to determine if protection against apoptosis is associated with changes in transcription factor binding activity. HUVEC were treated immediately post-irradiation with zinc pyrithione, a zinc ionophore, which rapidly and transiently elevates intracellular zinc levels. Zinc pyrithione treatment for 5 minutes blocked both IR-induced apoptosis and NFκB binding activity. Interestingly, all treatments with zinc pyrithione also decreased the amount of cytosolic IκB. Furthermore, all cells treated with zinc pyrithione exhibited decreased levels of basal AP-1 binding activity. IR-induced apoptosis was also inhibited by the protein synthesis inhibitor, cycloheximide. Thus, zinc pyrithione-induced protection against apoptosis is associated with a decrease in the binding activities of the transcription factors NFκB and AP-1. These results suggest that NFκB and AP-1, acting alone or in concert, may contribute to the induction of apoptosis in some systems.

Finally, we observed that increases in NFκB binding activity were associated with a potentiation of apoptosis. This was demonstrated by the fact that co-incubation of TPEN, an agent which induces apoptosis without activating NFκB, with TNFα, a cytokine which greatly elevates NFκB without effecting apoptosis in this model, significantly increased both NFκB binding activity and apoptosis. In summary, these studies suggest that the activation of NFκB contributes to the induction of apoptosis in HUVEC, and that both these processes can be inhibited by zinc pyrithione. Further investigation into the mechanisms of this zinc-induced protective effects seems warranted.

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LIST OF ABBREVIATIONS

Act D - actinomycin D
AP-1 - activator protein-1
CH - cycloheximide
Da - Daltons
DD - death domain
DED - death effector domain
DMSO - dimethyl sulphoxide
DNA - deoxyribonucleic acid
DTT - dithiothreitol
EDTA – ethylenediaminetetra-acetic acid disodium salt
EMSA - electrophoretic mobility shift assay
HUVEC - human umbilical vein endothelial cells
IκB - inhibitor of NFκB
ICE - interleukin 1β converting enzyme
I - ionizing radiation
IRF - interferon regulatory factor
LPS - lipopolysaccharide
NAC - N-acetylcysteine
NaCl - sodium chloride
NFκB - nuclear factor kappa B
NLS - nuclear localization sequence
PBS - phosphate buffered saline
PDTC - pyrrolidine dithiocarbamate
PMSF - phenylmethyl sulfonyl fluoride
RHD - rel homology domain
ROI - reactive oxygen intermediates
RT - room temperature
SDS - sodium dodecyl sulfate
SOD - superoxide dismutase
TAE - tris-acetic acid-EDTA
TBST - tris buffered saline with TWEEN-20
TE - tris-EDTA
TNFα - tumour necrosis factor alpha
TNF-R55 - 55kDa TNFα receptor
TNF-R75 - 75kDa TNFα receptor
TPEN - N, N, N' N'-tetrakis(2-pyridylmethyl)ethylenediamine
UV - ultraviolet
VCAM - vascular cell adhesion molecule

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INHIBITION OF APOPTOSIS AND TRANSCRIPTION FACTOR BINDING ACTIVITY BY ZINC PYRITHIONE IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

INTRODUCTION

1. APOPTOSIS

1.1 Apoptosis versus Necrosis

Cell death has been characterized as occurring by one of two ways: necrosis or apoptosis[1-4]. Necrotic cell death is passive, and occurs in response to a loss of cellular function and regulation resulting from severe trauma, whereas apoptotic cell death is active, and occurs in response to a signal that triggers a characteristic series of events in which the cell sets about to actively cause its own death. Necrosis is characterized by cell swelling and lysis due to the disruption of membrane integrity, resulting in the release of cellular contents. Necrosis is normally followed by an inflammatory response, as a result of the release of pro-inflammatory cellular agents into the surrounding tissue[1]. In contrast, apoptosis is a highly regulated form of cell death. Apoptotic cell death follows a predictable program of events including the expression of genes, the synthesis of proteins and the activation of molecules[5]. During apoptosis, the cytoplasm condenses, the cell shrinks and retracts from its neighbours and fragmentation of the DNA occurs. The cell then fragments into numerous apoptotic bodies that are phagocytozed by nearby cells or circulating macrophages. In contrast to necrotic cell death, apoptosis does not involve the release of intracellular contents and is not normally followed by an inflammatory response[6]. Necrotic cell death occurs in response to an external insult, while apoptotic cell death is physiologically signaled by either external or internal signals.

1.2 General Comments

The term apoptosis was first coined in 1972 by Kerr et al.[7] to describe this morphologically

distinct form of cell death. Apoptosis is derived from the ancient Greek and signifies a 'falling off', comparable to the loss of individual cells from a population[8]. Apoptotic cell death can occur in response to a variety of stimuli, both physiological and external. For example, if a cell's ultimate destiny is death, such as the interdigital cells present during early mammalian embryogenesis, apoptotic death is triggered by a naturally occurring and physiological signal. Similarly, other forms of embryological development, tissue remodeling, normal cell turnover, the functioning of the immune system and the development of the nervous system involve apoptosis triggered by normally occurring physiological signals[3,9-11]. In contrast to these physiologically occurring signals, apoptosis can also be triggered by external signals such as radiation[12-37], chemotherapeutic agents[20,38-42] or cytotoxic agents[43-45]. Furthermore, aberrations in the apoptotic pathway will lead to dysfunction and pathology[46-48]. Disruption of naturally occurring apoptotic cell death is associated with diseases such as cancers, auto-immune diseases and viral infections. In addition, the undesirable activation of apoptosis is associated with the pathogenesis of neurodegenerative diseases such as Alzheimer's and Parkinson's and of other diseased states such as AIDS.

Morphologically, apoptosis is characterized by cytoplasmic condensation and cytoskeletal disruption, cell shrinkage and detachment from neighbouring cells, plasma membrane blebbing, breakdown of the nuclear envelope, chromatin condensation and the formation of apoptotic bodies[4,7]. The leakage of cellular contents into the surrounding areas is prevented by the formation of apoptotic bodies and as such, apoptosis is not normally followed by an inflammatory response. Neighbouring cells or circulating macrophages then remove the apoptotic bodies by phagocytosis[2,7]. Biochemically, apoptosis is generally characterized by the distinct fragmentation pattern of the DNA[10]. Apoptotic DNA fragmentation results from the activation of an endogenous and topographically constrained endonuclease. The activated endonuclease generates double stranded DNA cuts between nucleosomes, resulting in DNA fragments that are multiples of 180-200 base pairs. When viewed on an agarose electrophoretic gel, these fragments appear as 'ladders'[9,49]. While DNA laddering has long been considered a hallmark of apoptosis, it is not

essential and examples of apoptosis occurring in the absence of DNA laddering have been reported[50-52]. During apoptosis, endonucleases may also cut DNA into 50kb fragments prior to internucleosomal cleavage[53,54]. Other biochemical events that may occur include the externalization of phosphatidyl serine residues in the plasma membrane[55], the accumulation or activation of transglutaminase in the cytoplasm[56,57], cellular acidification[58-60] and a reduction in mitochondrial transmembrane potential[61,62].

1.3 Apoptotic Signaling

Apoptosis is a highly regulated and controlled form of cell death. However, the actual mechanisms regulating the apoptotic pathway have yet to be fully elucidated. Extracellular agents capable of inducing apoptosis include cytokines such as tumour necrosis factor alpha (TNFα)[14,63-71] and Fas ligand[72,73], ultraviolet (U.V.)[45,74-76] and γ-irradiation[12-37], oxidative stress[77-85] and chemotherapeutic agents[20,38-42]. The transduction of these extracellular signals to intracellular agents results in apoptosis. The intracellular agents involved in the signaling of apoptosis include phosphatases [86,87], kinases[23,28,29,34,87-93], proteases[94-97], calcium[32,49,90,98-101] and zinc[5,49,74,102-109] ions, and the interaction of these agents with gene products involved in the regulation of apoptosis. In this section, focus will be placed on the induction of apoptosis by the extracellular agents relevant to this study.

1.3.1 Oxidative Stress

Oxidants have recently become recognized as widely utilized signaling molecules[110]. Indeed, there is substantial evidence implicating the involvement of oxidative stress in apoptosis. The addition of reactive oxygen intermediates (ROI), such as H_2O_2, or of agents which induce the formation of ROI, such as γ-irradiation, TNFα, and transition metals (through the Fenton reaction), have been shown to induce apoptosis in a variety of cell and tissue types[65,80,83,111,112]. Additionally, the depletion of endogenous antioxidants such as glutathione or Cu/Zn superoxide dismutase (SOD) results in apoptosis or an increased

susceptibility to apoptosis[82,83,113]. Thus, apoptosis is induced by stimuli that increase oxidative stress within a cell, either by increasing the level of ROI or by depleting the endogenous antioxidants. Further supporting a role for oxidative stress in the induction of apoptosis is the protection provided by antioxidants against a wide variety of apoptosis inducers. Antioxidants or free radical scavengers such as N-acetylcysteine (NAC), thioredoxin, MnSOD, pyrrolidine dithiocarbamate (PDTC) or 1,10-phenanthroline have been shown to protect against apoptosis induced by glucocorticoids, γ-irradiation, etoposide and TNFα[65,111,112,114-118]. Oxidative stress has been suggested to be a final common step in the convergence of pathways signaling apoptosis[78]. However, the ability of cells to undergo apoptosis in an anaerobic environment and the ability of agents, such as Fas ligand to induce apoptosis in the absence of ROI appears to refute that theory[119].

1.3.2 Ionizing Radiation

Ionizing radiation has been shown to induce apoptosis in a variety of cell and tissue types. Cells exposed to ionizing radiation (IR) will undergo cell cycle arrest, the activation of DNA repair mechanisms and a reduction in DNA synthesis[14,22]. IR has also been shown to induce gene transcription and the expression of new proteins[13,33,35,120-123]. However, when significant damage has been sustained, IR has been shown to induce apoptosis[14,19,22,23,27-29,33,92,124-128]. The mechanisms by which IR induces apoptosis have yet to be resolved, although several events initiated by IR will contribute to the induction of apoptosis. IR has been shown to directly induce DNA damage, to generate the formation of free radicals and to alter membrane structure[14], all of which can contribute to apoptotic cell death.

The generation of single-stranded and double-stranded DNA breaks by IR appears to induce a p53-dependant apoptotic pathway. The transcription factor p53 induces cell cycle arrest at the G1 phase, allowing for the detection of DNA damage and the initiation of DNA repair post irradiation. However, when irreparable damage has been sustained, p53 will mediate the induction of apoptosis[129-131]. The critical

involvement of p53 in the induction of IR-induced apoptosis is supported by the observation, among others[24,132,133], that thymocytes deficient in p53 will not undergo apoptosis in response to IR[130]. It has been suggested that p53 may induce the expression of bax, a promoter of apoptosis, while suppressing the expression of the anti-apoptotic protein bcl-2[134,135]. Interestingly, IR-induced apoptosis in lymphocytes appears to involve the activation of another transcription factor, interferon regulatory factor (IRF)-1, which in turn induces the activation of yet another apoptotic-effector protein, interleukin-1β-converting enzyme (ICE). Thus, IR-induced DNA damage appears to induce apoptosis by the activation of gene transcription.

The generation of ROI induced by IR may also contribute to apoptotic death[19,78]. Exposure to IR causes the immediate conversion of water to hydrogen and hydroxyl free radicals in the cell. These ROI may then contribute to the induction of apoptosis either by damaging DNA or by imposing a state of oxidative stress within the cell. Interestingly, it has recently been suggested that p53-mediated induction of apoptosis may occur via the selective transcription of oxidative stress-inducing genes by p53[136].

Alternatively, IR-induced apoptosis may also be signaled by perturbations in the plasma membrane[28,137]. Indeed, incubation of cells with the lipophilic vitamin E derivative Trolox was found to prevent IR-induced apoptosis[137,138]. IR, acting on the cellular membrane, can also induce the hydrolysis of sphingomyelin to ceramide[28]. Ceramide has been shown to be a powerful inducer of apoptosis[139,140], and it has been suggested that the generation of ceramide following IR is the most important mechanism by which IR-induced apoptosis is signaled[28].

Thus, DNA damage, oxidative stress or perturbations of the plasma membrane may signal IR-induced apoptosis. The apparent requirement for gene transcription in models of IR-induced apoptosis is intriguing and bears noting.

1.3.3 TNFα

Recent evidence suggests that the induction of apoptosis by TNFα is regulated by the interaction

of two signaling pathways, both induced by TNFα binding to cell surface receptors[68]. One pathway appears to induce apoptosis by the recruitment of adapter molecules which signal cell death in the absence of protein synthesis, while the other pathway appears to protect against apoptosis by inducing the synthesis of protective proteins[68,141]. The evidence suggests that TNFα induces apoptosis by binding and clustering the 55kD TNFα receptor (TNF-R55). The intracellular domain of the TNF-R55 contains an approximately 90 amino acid sequence, termed the death domain (DD) near the C-terminus[142]. The interaction of the receptor DD with the DD of adapter molecules results in considerable cross-talk with the Fas receptor and appears to initiate the signal transduction pathway involved in apoptosis. The DD of the TNF-R55 associates with the adapter protein TRADD[66], which in turn can associate with the Fas ligand-induced Fas associated protein MORT1/FADD[143,144], also via the DD[141]. The region upstream of the MORT1/FADD DD is required for both Fas ligand and TNFα (through TNF-R55)-induced apoptosis[145]. Additionally, both TRADD and MORT1/FADD can interact with RIP[146-148], another signaling molecule involved in the induction of apoptosis. It appears as though the recruitment of these adapter molecules and others are responsible for the activation of various members of the caspase family of proteases, which appear to be involved in the effector stage of apoptosis (caspases are discussed in more detail in section 1.4). Briefly, caspases -8 and -10 contain death effector domains (DED) or MORT domains which will associate with DED located upstream of the DD in MORT1/FADD[141,149,150], while caspase-2 interacts with RAIDD/CRADD[151,152] which will associate with RIP through DD. Thus, substantial evidence suggests that the recruitment of adapter molecules by the DD of clustered TNF-R55 initiates a series of events responsible for TNFα-induced apoptosis[68,141,153].

However, TNFα has also been shown to induce significant amounts of ROI production in various cell types [83,154,155], and apoptosis induced by TNFα can be inhibited by treatment with antioxidants . NAC, PDTC and bcl-2 have all been shown to protect against TNFα-induced apoptosis in different cell types[64,65,156]. This suggests that in addition to a direct activation of an apoptotic-signaling cascade initiated

by interactions of the DD of TNF-R55 with adapter molecules, TNFα may also induce apoptosis by the formation of ROI.

Paradoxically, the activation of the TNF-R55 can also induce a pathway that requires protein synthesis, and which appears to be involved in the protection against apoptosis[68]. The adapter molecule TRADD can also associate with TRAF2[157], which appears to initiate a signaling cascade culminating in the synthesis of apoptosis-suppressing proteins[68]. The TRAF2 pathway is also signaled by TNFα binding to the 75 kD TNFα receptor (TNF-R75)[158]. The nature of the protection afforded by the activation of these pathways is still under investigation, although it may involve the transcription factor Nuclear Factor kappa B (NFκB)[20,127,159,160]. However, the role of NFκB in apoptosis is still controversial (see section 2.2.3).

The apparent ability of TNFα to both induce and block apoptosis is reflected in the effects of TNFα on various cell types. TNFα has been widely reported to induce apoptosis in both primary and transformed cell types[63-65,127,161-163]. However, others have shown that treatment with TNFα alone does not induce apoptosis[70,83,139,156,159,164]. In both cases, co-treatment with protein synthesis inhibitors has been shown to either exacerbate[63] (if TNFα treatment alone induced apoptosis) or induce[70,156] (if TNFα treatment alone had no effect on apoptosis) apoptosis. Thus, the effect of TNFα appears to be complex and cell-dependent, involving the apparent interaction between the TNF-R55 and the TNF-R75-associated signal transduction pathways, and possibly involving an oxidative stress-induced response.

1.3.4 Chelation of Intracellular Zinc

Several groups have implicated the chelation of intracellular zinc in the induction of apoptosis[5,102,103,165,166]. Studies performed in the laboratory of Orrenius have shown that treatment of rat and human thymocytes with the membrane permeant heavy metal chelator N, N, N' N'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN) induced significant apoptosis, which was prevented by the addition of exogenous zinc[102,103]. Further studies performed by Jiang et al.[165] suggest that the induction of

apoptosis by TPEN involves a signaling pathway initiated in the cytoplasm and transduced to the nucleus. This was supported by the following observations: Firstly, they found that treatment of isolated nuclei with TPEN did not induce apoptotic morphology. Secondly, they found that the cytosolic fraction from TPEN-treated cells would induce apoptosis in isolated nuclei. Thirdly, they found that the addition of TPEN to the cytosol of untreated cells would not induce apoptosis in isolated nuclei. Thus, the authors suggested that TPEN-induced apoptosis involves a pathway requiring signaling transduction between the cytoplasm and the nucleus.

1.4 Gene Regulation of Apoptosis

It appears that all cells contain genes encoding apoptosis-suppressing and apoptosis-inducing factors. Accordingly, the regulation of apoptosis appears to depend on the relative expression of these apoptosis-suppressing and apoptosis-inducing factors. Insight into the nature of the genes responsible for these factors came from studies performed with the nematode *Caenorhabditis elegans*[167]. The expression of the *C.elegans* death genes, ced-3 and ced-4, were found to induce apoptosis, while the expression of ced-9 was found to suppress apoptosis[168-170]. The mammalian homologues of ced-9 include the members of the bcl-2 family of apoptotic regulators[171], while the ced-3 protein was found to share significant homology with ICE[172]. Members of the bcl-2 family of apoptotic regulators include the proteins Bcl-xL, Bcl-W, A1 and Mch1, which are involved in the inhibition or suppression of apoptosis, while other members, including the proteins BaX, Bik, Bad, and Bcl-xS are involved in the promotion of apoptosis[173,174]. The nature of the anti-apoptotic effect induced by Bcl-2 appears to be related to its cellular localization: the outer mitochondrial membrane, the endoplasmic reticulum and the outer nuclear membrane[175-178]. It has been suggested that Bcl-2 inhibits apoptosis by regulating the mitochondrial transmembrane potential[175]. In view of the marked ability to protect against oxidative stress-induced apoptosis, Bcl-2 may also exert antioxidant properties[179]. Bax has been shown to form heterodimers with Bcl-2, and in this manner, inhibit

the protection afforded by Bcl-2 against apoptosis. Thus, the ratio of Bcl-2 to Bax is an important determinant of the susceptibility of the cell to apoptosis[180,181]. Other apoptosis-inducing factors belong to the caspase family, which include ced-3 and ICE. The term caspase designates a group of cysteine proteases which cleave at aspartate residues[182]. Numerous caspase family members have been identified and are implicated in the effector stage of apoptosis. Caspases induce the proteolysis of a number of substrates, and appear to function in the disassembly of structural components, the inactivation of cellular repair and defense mechanisms, and the propagation of apoptotic signaling by the proteolysis and activation of other family members, among others[183]. While the caspase and bcl-2 gene families provide insight into highly conserved regulation of the apoptotic pathway, a host of other mammalian genes involved in the intracellular regulation of apoptosis have been identified. The expression of other genes, such as p53 and c-myc[184] are also implicated in the induction of apoptosis. In addition, the viral gene products of crmA[65,185,186] and p35[14,187] inhibit apoptosis by interfering with caspase activity.

In summary, since all cells appear to contain genes encoding both apoptosis-inducing and apoptosis-suppressing factors, it seems likely that susceptibility to apoptosis is regulated by the relative expression of these genes, which may in turn be stimulated or inhibited by apoptosis-inducing signals.

1.4.1 Role of Transcription and Translation

Early studies suggested that apoptosis required the de novo synthesis of apoptosis-inducing proteins[32,33,49,188-190]. Indeed, numerous studies have shown that the inhibition of macromolecular synthesis by various protein synthesis inhibitors, such as cycloheximide (CH)[32,33,190], or by the transcription inhibitor actinomycin D (Act D)[32,33,190], inhibits apoptosis induced by glucocorticoids, γ-irradiation, growth factor removal, and Ca^{2+}-ionophores. However, in recent years it has become apparent that the regulation of apoptosis is far more complex. For example, inhibitors of macromolecular synthesis have since been shown to be ineffective in protecting certain cells against apoptosis[102]. Furthermore, these inhibitors have

been shown to both potentiate and induce apoptosis in various instances[41,45,63,70,105,156,191]. In these cases, it has been suggested that the necessary machinery for the induction of apoptosis is already present in the cell, and is kept in check by the continuous transcription and/or translation of apoptosis-suppressing factors[192]. The apparent contradictory effects of the inhibition of macromolecular synthesis on apoptosis emphasize the complexity of this event.

Since apoptosis is regulated by the relative expression of apoptosis-inducing and apoptosis-suppressing factors, the expression and stability of these proteins is likely to regulate the induction of apoptosis. Accordingly, apoptosis may be signaled by the activation of transcription factors which regulate the expression of apoptosis-inducing factors. Apoptosis may therefore be controlled by the activation of transcription factors involved in the regulation of apoptosis-suppressing or apoptosis-inducing genes.

2. TRANSCRIPTION FACTORS

2.1 General Comments

One of the mechanisms by which the expression of genes within a cell is regulated is through the regulation of transcription. The transcription of genes is in turn regulated by the binding of trans-acting factors to the promoter and enhancer regions of the gene. The trans-acting factors, or transcription factors, activate or repress the transcription of genes, thus affecting gene expression. Transcription factors are proteins which are capable of entering the nucleus and binding to DNA. They contain peptide regions and structural motifs which display high affinity to selective segments (binding sites) of DNA. This binding occurs mainly through hydrogen bonds, ionic bonds and hydrophobic interactions. Thus, apoptosis may be signaled through the modulation of transcription factor binding, which will in turn alter gene expression such that the balance of apoptosis-inducing and apoptosis-suppressing factors is shifted in favor of apoptosis. In this section, focus will be placed on the transcription factors relevant to this study. Of

particular interest are the redox regulated transcription factors NFκB and Activator Protein-1 (AP-1). Also of interest is the zinc finger containing transcription factor Sp1.

2.2 Nuclear Factor kappa B

The NFκB family of transcription factors is composed of dimerized subunits of Rel-family proteins[193-195]. The members of the Rel-family proteins that comprise NFκB are p50, p52, Rel A (p65), c-Rel and Rel B. The NFκB subunits are characterized by a Rel homology domain (RHD), which contains a DNA binding domain, a nuclear localization sequence (NLS) and a dimerization domain. The NFκB subunits will all dimerize to form homodimers or heterodimers, with the exception of Rel B, which does not form homodimers, and will form heterodimers only with p50 and p52. The 'classic' and widely studied NFκB consists of p50/p65[193]. The formation of distinct NFκB dimers permits differential regulation of gene expression by altering both the DNA binding properties of NFκB and the interactions of NFκB with other transcriptional regulatory proteins[196-198]. Thus, the regulation of gene expression by NFκB is in part determined by the subunits that comprise the transcription factor. The activation of the transcription factor NFκB occurs independently of protein synthesis[199]. Prior to induction, the preformed NFκB is maintained in the cytoplasm in a latent, inactivated state by an inhibitor of NFκB (IκB) protein [200]. NFκB is activated as a result of the sequential phosphorylation, ubiquitination and proteosome-mediated degradation of IκB and subsequent translocation of NFκB to the nucleus[201-205]. The phosphorylation of serines 32 and 36[206] by a kinase signals the ubiquination of IκBα, which in turn targets the degradation of IκBα by proteosomes. The signal transduction pathways leading to IκB degradation and subsequent NFκB translocation have yet to be fully elucidated, and evidence has been presented supporting a role for kinases, phosphatases, ROI and the redox state[203,207,208]. Members of the IκB family of proteins include IκBα, IκBβ, IκBϵ and IκBγ, and are characterized by the presence of between 5-7 ankyrin repeats in their internal region[209-212]. The ankyrin repeats interact with the RHD of the NFκB subunits and block the NLS,

preventing the nuclear translocation, and hence the activation, of NFκB[213]. The precursors to the p50 and p52 subunits, NFκB1 and NFκB2, respectively, also contain ankyrin repeats and function as IκB proteins by inhibiting the nuclear translocation of NFκB[214,215]. Thus, cytosolic forms of NFκB include mature dimers bound to an IκB protein, or monomers bound to NFκB1 or NFκB2. Different forms of NFκB appear to preferentially associate with a specific IκB protein. For example, dimers consisting of c-Rel or p65 are often associated with IκBα[216]. The last member of the IκB family, Bcl-3, binds p50 or p52 homodimers, and in contrast to the other members of the IκB family, is localized in the nucleus where it functions as a transcriptional activator[217,218].

The transcription factor NFκB can be rapidly activated in response to a variety of stimuli and can in turn regulate the expression of numerous genes by binding to their promoter or enhancer regions. NFκB is activated by stress or injury inducing stimuli, such as the inflammatory cytokines TNFα[64,139,164,219-226] and IL-1[225,227-229], lipopolysaccharides (LPS)[203,221,230], agents inducing oxidative stress [164,231-238], UV[193,234,239] and γ-irradiation[20,122,239-243], viral or bacterial infections and B or T cell activation[193,203,244]. Activated NFκB targets genes involved in mediating inflammatory, immune and acute phase responses, such as cytokine and cytokine receptor genes[203,245], endothelial activation genes[226,246-250] and others. The inhibitory protein, IκBα, also contains NFκB binding sequences in its promoter region, and is rapidly upregulated following the induction of NFκB[251]. Interestingly, NFκB binding sequences have also been located in the promoter regions of the protooncogenes c-myc[252] and p53[253]. In the following section, focus will be placed on the activation of NFκB by agents relevant to this study. Subsequent sections will focus on the DNA binding of NFκB and the involvement of NFκB in apoptosis.

2.2.1 Activators of NFκB

Activation of NFκB has been reported following treatment of various cell types with agents that induce oxidative stress[164,240,254], IR[20,239-243] and TNFα[64,139,164,219-226].

2.2.1.1 Oxidative Stress

The activation of NFκB has been shown to occur by stimuli that induce oxidative stress[164,240,254]. The addition of ROI, such as H_2O_2[164,224,232,236,255,256] or HOCl[257], or the generation of oxidative stress by agents that induce the formation of ROI, such as IR[240] or TNFα[224,258], and reoxygenation after hypoxia[235] or hyperoxia[259], has been shown to induce the activation and nuclear translocation of NFκB in numerous cell types. ROI-induced activation of NFκB is associated with the degradation of IκB[224,257,260]. The ability of antioxidants to inhibit the activation of NFκB by diverse inducers further supports the involvement of ROI in the activation of NFκB. The antioxidants NAC, PDTC, metal chelators and vitamin E and its derivatives have all been shown to inhibit the activation of NFκB by various stimuli including H_2O_2, inflammatory cytokines, PMA and LPS[226,230,231,249,254,257,261,262]. Antioxidants appear to inhibit the activation of NFκB by preventing the phosphorylation of IκB[224]. However, the signal transduction pathways leading to the degradation of IκB and activation of NFκB have yet to be elucidated. Thus, ROI appear to play a critical role in the activation of NFκB by various inducers, and it has been suggested that ROI serve as a common mediator in the transduction pathways signaling the activation of NFκB.

2.2.1.2 Ionizing Radiation

Several cell types have been shown to induce the activation of NFκB following exposure to IR[20,239-243]. The activation of NFκB following exposure to 200-5000 Rads of IR in human myeloid leukemia cells has been reported[242], while other have shown that lower doses (10-200Rads) will activate NFκB in human lymphoblastoid cells[240]. These authors have shown that the activation of NFκB was inhibited by NAC[240]. Thus, the generation of intracellular ROI by IR, and the ability of NAC to inhibit the induced NFκB, suggests that ROI may mediate the activation of NFκB by IR.

2.2.1.3 TNFα

TNFα has been shown to induce the activation and nuclear translocation of NFκB in a wide variety of cell types [64,65,139,164,219-226]. The emerging evidence suggests that TNFα induces the degradation of IκB and subsequent activation of NFκB by a cascade of events initiated by the binding and clustering of the 75kD TNFα receptor (TNF-R75). The cascade involved includes the recruitment of the adapter protein TRAF2[158], which, when overexpressed, has been shown to signal the activation of NFκB[263]. As TRAF2 has been shown to associate with TRADD, the activation of NFκB may also be signaled by TNFα binding to TNF-R55[68,157]. However, TNFα has also been shown to increase intracellular ROI[154,224], and in several instances, antioxidants have been reported to inhibit the TNFα-induced activation of NFκB[65,226,230,245,258,264]. Thus, the activation of NFκB by TNFα may also involve the generation of ROI.

Thus, substantial evidence implicates ROI as an intracellular mediator in the activation of NFκB. However, in vitro studies have shown that the DNA binding of NFκB is sensitive to oxidation, and requires a critical cysteine residue.

2.2.2 DNA binding of NFκB

In vitro studies investigating the DNA binding ability of purified NFκB have identified, by mutational analysis, a critical cysteine residue. The sulfhydryl group of cysteine 62 was found to be required for DNA binding of NFκB[265]. Indeed, others had previously shown that the in vitro binding of NFκB was inhibited by oxidants[266], suggesting that the oxidation of critical cysteines decreased the DNA binding ability of NFκB. Additionally, it has been shown that the modification of free sulfhydryls in NFκB by alkylating or oxidizing agents inhibits the in vitro binding of NFκB to DNA consensus sequences[266]. Taken together, it appears as though the oxidation state of sulfhydryls, in particular the sulfhydryl residue of the critical cysteine 62, is an important factor in the regulation of DNA binding by NFκB in vitro. It follows that modification of this cysteine residue may also play an important role in the regulation of

NFκB binding in vivo.

Interestingly, the DNA binding ability of NFκB also appears to be dependent on zinc. Zinc has been reported to be required for the binding of purified NFκB, while high concentrations of zinc inhibit the binding of purified NFκB in vitro[267]. Thus, there appears to be an optimal level of zinc required for NFκB binding. The ability of zinc to interact with the sulfhydryl group of cysteine residues may be responsible for the effects of zinc on NFκB binding ability.

2.2.3 The NFκB-Apoptosis Paradox: Inhibition or Stimulation?

The role of NFκB in the regulation of apoptosis is controversial[260]. Arguments and evidence have been presented suggesting that NFκB can be involved in either the induction or the prevention of apoptotic cell death. Several arguments supporting a role for NFκB in the induction of apoptosis have been made. NFκB is induced by numerous stimuli that also induce apoptosis, and the inhibition of apoptosis induced by these stimuli is often accompanied by a decrease in NFκB activation or binding[179,268]. Additionally, NFκB binding sequences have been located in the promoter regions of several genes involved in the induction of apoptosis. The promoter regions for p53[253,268], c-myc[252], murine ICE[269], Fas[270] and c-Rel[268] all contain NFκB binding sequences. Evidence has also been presented supporting a requirement for NFκB in the induction of apoptosis by several different stimuli. Serum-deprivation was found to both induce apoptosis and activate NFκB in a human kidney cell line. Transfection of these cells with a mutant p65 subunit of NFκB lacking the transactivating domain partially inhibited the serum-deprivation-induced apoptosis[268]. Truncated versions of other NFκB subunits lacking the transactivating domain have also been shown to inhibit IR-, Ca^{2+} ionophore- and serum deprivation-induced apoptosis[271]. The mutation of IκBα, such that the activation of NFκB was prevented, reduced the IR-induced apoptosis in fibroblast cells[272]. Others have demonstrated a requirement for NFκB activation in the virally-induced apoptosis of a prostate carcinoma cell line[273]. Thus, it appears as though NFκB may regulate the induction of apoptosis, at least in

some cell types. However, it has also been suggested that the activation of NFκB protects against apoptosis.

Several studies have also demonstrated that the activation of NFκB confers protection against apoptosis. Mice lacking the p65 subunit undergo massive apoptosis in the liver, suggesting that the expression of p65 is required to prevent cell death[274]. Indeed, others have shown that mouse fibroblasts deficient in p65 undergo increased cell death in response to TNFα, as compared to wild-type fibroblasts[159]. Additionally, the generation of 'super repressor' forms of IκB or IκB mutants, both of which prevent NFκB translocation, have been shown to sensitize cells to apoptosis induced by TNFα, IR, and chemotherapeutic agents[20,127]. The transfection of vectors containing p65 into the p65-deficient fibroblast or of p65 and p50 into the IκB 'super repressor' cells prevented the observed sensitization to the induced apoptosis. Consistent with the apparent apoptosis suppressing role of NFκB, the induction of an anti-apoptotic protein, A20, has also been reported following NFκB activation, and appears to be dependent on the translocation of the p65 subunit[70,159,275]. Additionally, it has recently been reported that NFκB is involved in the regulation of c-IAP2, an inhibitor of apoptosis gene[71]. Hence, a protective role for NFκB has also been established.

Thus, while the activation of NFκB has been reported to be required for the induction of apoptosis, contrasting evidence has also been presented demonstrating a protective role for NFκB during apoptotic cell death. The apparent contradictory functions of NFκB may be reconciled by the nature of this transcription factor. The subunit composition of NFκB may influence the binding of NFκB to either pro- or anti-apoptotic genes. In turn, the stimulus inducing apoptosis may selectively activate different subunits in different cell types, or alternatively, the same subunits may be activated but with different effects depending on the cell type or stimulus. Thus, the activation of either pro- or anti-apoptotic genes by NFκB may be cell-type dependent. In addition, it has been well established that cell-type specific effects of NFκB require the presence of additional factors. For example, while NFκB binding sequences have been

located in the promoter region of the vascular cell adhesion molecule (VCAM-1), the expression of VCAM-1 requires the additional presence of another transcription factor [276]. Similarly, interactions between NFκB and the transcription factor AP-1 are required, in some instances, for the activation of gene expression[277]. Thus, NFκB may exert either a pro- or anti-apoptotic effect depending on the presence or activation of other transcription factors that synergize with NFκB to regulate apoptosis. This in turn may also be dependent on both the cell-type and stimulus. Hence, NFκB may differentially regulate apoptosis by the induction of apoptosis promoting or apoptosis suppressing genes, depending on the subunit composition of NFκB, the synergistic effects of NFκB with other transcription factors, and the cell-type and stimulus-type inducing the apoptosis.

2.3 Activator Protein-1

The AP-1 family of transcription factors is composed of dimerized subunits of Fos and Jun[278]. The gene products of the proto-oncogenes fos and jun contain hydrophobic regions which interact during dimerization to form a "leucine zipper". All Fos and Jun proteins will dimerize to form heterodimers, but only Jun proteins will form homodimers[278]. The activation of the transcription factor AP-1 is induced by the novel synthesis of AP-1 subunits or by the activation of pre-existing AP-1 dimers. The induction of AP-1 normally involves the expression of Fos/Jun containing heterodimers, while Jun homodimers are generally present in a steady state[279]. Fos/Jun heterodimers exhibit increased binding activity as compared to Jun homodimers[280]. As with NFκB, the formation of distinct AP-1 dimers permits differential regulation of gene expression.

The transcription factor AP-1 is induced in response to a variety of stimuli and can in turn regulate the expression of numerous genes. AP-1 is activated by phorbol esters[281-283], cytokines such as TNFα[164], ROI[164,255,283,284], as well as antioxidants[245,261] and others[235,285]. Targets for AP-1 activation include c-jun[286], growth-factor-inducible genes[287], stress-induced genes[288]. The following section will focus on the

activation of AP-1 by agents relevant to this study. Subsequent sections will focus on the DNA binding of AP-1 and the involvement of AP-1 in apoptosis.

2.3.1 Inducers of AP-1

The activation of AP-1 by various agents has been attributed to the formation of ROI. Treatment of cells with ROI, such as H_2O_2[164,255], or agents that induce the formation of ROI, such as TNFα[164,289] or IR[290,291], has been shown to activate AP-1. Cells treated with IR have been shown to induce both the expression of c-fos and c-jun and the activation of AP-1[124,290-292], while similar results have been reported with cells treated with TNFα, where TNFα was shown to activate the DNA binding ability of AP-1 and the expression of c-fos and c-jun[164,293-297]. The ability of antioxidants to suppress various forms of ROI-induced AP-1 activation suggests the involvement of a ROI dependent pathway in the activation of AP-1[234]. Thus, various agents that induce ROI will also activate AP-1, and this activation can be attributed to the formation of ROI. Paradoxically, treatment with antioxidants alone has also been shown to greatly induce AP-1. Recent evidence indicates that a wide range of antioxidants will induce AP-1 activation to an even greater extent than ROI[279]. Treatment of cells with NAC, PDTC and other antioxidants has been shown to greatly enhance the DNA binding ability of AP-1 and has been associated with an increase in gene expression[245]. Thus, treatment of cells with either pro-oxidants or antioxidants appears to signal the expression of AP-1 subunits and AP-1 activation. In addition, in vitro studies on the DNA binding ability of AP-1 have shown that AP-1 requires a critical cysteine residue and is sensitive to oxidation[278].

2.3.2 DNA Binding of AP-1

The DNA binding domain of AP-1 is generated by the dimerization of AP-1 subunits. Within the DNA binding domain there exists a highly conserved cysteine residue essential for the binding of AP-1 to DNA concensus sequences[278]. In vitro studies have shown that oxidation of this critical cysteine residue

inhibits the DNA binding ability of AP-1, which can be restored upon subsequent treatment with reducing agents[266]. The DNA binding ability of AP-1 in vitro is also increased by treatment with reducing agents alone[298]. Thus, modification of the critical cysteine residue by oxidation inhibits the DNA binding ability of AP-1, suggesting that other agents also capable of interacting with cysteine residues may also alter the DNA binding ability of AP-1.

2.3.3 Possible Involvement of AP-1 in Apoptosis

The activation of AP-1 occurs in response to a variety of stimuli that also induce apoptosis[299-302]. Thus, it has been suggested that AP-1 may play a role during apoptotic cell death. Further investigations in this area have revealed a requirement for AP-1 in certain types of cell death[303], while others suggest that AP-1 is not essential for apoptosis to occur[304,305].

Early studies linking apoptosis and AP-1 demonstrated an increase in c-fos and c-jun expression by stimuli that also induced apoptosis. Indeed, AP-1 activation has been shown to occur in response to numerous agents, including ceramide[303], which also induces apoptosis[128]. Furthermore, apoptosis induced by ceramide was found to be inhibited by agents such as curcumin, which inhibits AP-1 binding, or by anti-sense c-jun mRNA[303]. Others have also reported a requirement for c-jun in the induction of apoptosis by various other agents[306,307]. Thus, AP-1 appears to be implicated in the apoptotic response to certain stimuli. However, other studies suggest that AP-1 is not required for apoptosis. During development and in isolated cell systems, apoptosis has been shown to occur in the absence of c-Fos or c-Jun[308,309], while apoptosis induced by TNFα or Fas does not require the activation of AP-1[304,305].

The full involvement of AP-1 in apoptosis has yet to be resolved. The involvement of AP-1 in apoptosis may be stimulus-dependent. The requirement for AP-1 in the induction of apoptosis by certain agents suggests the targeting of pro-apoptotic genes by AP-1. However, potential apoptotic genes regulated by AP-1 have yet to be identified. In addition, AP-1 transactivating potential may be influenced

by the presence of synergizing transcription factors. Thus, apoptosis requiring the activation of AP-1 may also require the presence of other factors that synergize with AP-1 to promote apoptosis[310]. In summary, then, the involvement of AP-1 in apoptosis appears to be stimulus-dependent, and likely involves the transactivation of as yet unidentified, apoptosis-promoting genes. Other transcription factors acting synergistically with AP-1 may also influence apoptosis.

2.4 Sp1

Sp1 is a member of a large family of transcription factors that bind preferentially to nucleotides sequences rich in guanine and cytosine[311]. Sp1 is composed of three "zinc fingers", and as such contains essential cysteine residues required for binding zinc and forming the DNA binding motif[312]. Sp1 is expressed in most tissues and can bind with varying affinities to consensus sequences rich in guanine and cytosine. Sp1 often binds to genes lacking TATA or CAAT boxes in the promoter region[313] and has been shown to regulate numerous genes in diverse classes, including housekeeping genes, inducible genes and viral genes[314-316]. DNA binding of Sp1 is inhibited by oxidation, both in vitro and in situ[313,317]. The inhibition of Sp1 binding by oxidation can be attributed to the oxidation of cysteine residues involved in the formation of zinc fingers and can in turn be reversed by the antioxidant, dithiothreitol (DTT)[313]. Modification of cysteine residues with alkylating agents will also inhibit the DNA binding of Sp1 [313]. Interestingly, the chelation of zinc with thionein has been shown to decrease Sp1 binding activity [318]. To date, there is no direct evidence implicating Sp1 in the regulation of apoptosis.

Thus, there is presently substantial evidence suggesting a role for the transcription factors NFκB and AP-1 in the regulation of apoptosis. Factors that influence the binding of these transcription factors to their consensus sequences may in turn modulate apoptosis. The ability of zinc to interact with cysteine residues and the dependence of NFκB and AP-1 on critical cysteine residues suggests that zinc may modulate apoptosis by influencing the DNA binding activity of these transcription factors. Indeed, zinc

has been shown to modulate apoptosis in a variety of systems.

3 ZINC

3.1 Functions of Zinc

Zinc is an essential trace element and is required for many cellular functions. Hundreds of zinc-containing enzymes have been identified in which zinc functions as a structural component or as a required component of the catalytic activity. Zinc is also responsible for the maintenance and stabilization of some structural conformations, such as zinc fingers, which are required for transcription factor binding to DNA[319]. In addition, zinc plays a structural role in numerous other proteins and in the interactions of proteins with other macromolecules[320]. The ability of zinc to bind to the sulfhydryl groups of cysteines often mediates these effects[320,321]. In addition to structural and enzymatic functions, zinc has also been shown to block the generation of free radicals[322] and to act directly as an antioxidant[323] within the cell. Interestingly, it has also been suggested that zinc may possibly function as a signaling molecule[320]. The level of zinc within a cell may affect protein interactions and gene expression by modulating signal transduction pathways or molecules involved in signal transduction. Despite the essential nature of zinc, little is known concerning the regulation of zinc within the cell.

3.2 Zinc and Apoptosis

Zinc inhibits apoptosis induced by a variety of agents. High concentrations of zinc have been shown to prevent the DNA fragmentation associated with, among others, the glucocorticoid[49,324]-, IR[19,33,324], TNFα[325]- and etoposide[326]-induced apoptosis in numerous cell types. Indeed, the ability of zinc to prevent DNA fragmentation has been used to verify that apoptotic cell death had occurred. In addition, cells deficient in zinc[105,108] have been shown to undergo apoptosis and the chelation of intracellular zinc[102,103] has

been implicated in the induction of apoptosis in different cell types. Thus, high concentrations of zinc are capable of suppressing induced apoptosis, while the removal of zinc from normal systems can induce apoptosis. However, in some systems, treatment with zinc has proven ineffective in the prevention of cell death[327,328]. The ability of zinc to inhibit apoptosis has, until recently, been assumed to be attributed to the inhibition of the Ca^{2+}/Mg^{2+}-sensitive endonuclease[49,109,329]. However, recent reports suggest that zinc may function elsewhere in the cell to inhibit apoptosis.

3.2.1 Inhibition of the Endonuclease by Zinc

Initial studies performed by Cohen and Duke[49] demonstrated that zinc is a powerful inhibitor of the Ca^{2+}/Mg^{2+}-sensitive endonuclease responsible for DNA fragmentation during apoptosis. Since then, zinc has been shown to inhibit apoptosis in a variety of systems, and the inhibition of apoptosis has largely been attributed to the prevention of DNA "laddering", induced by the fragmentation of DNA. However, zinc has also been shown to prevent apoptosis in cells that appear to lack a Ca^{2+}/Mg^{2+}-sensitive endonuclease[330], and to inhibit signs of apoptosis that occur prior to DNA fragmentation[59]. Thus, it has been suggested that zinc may function elsewhere in the cell to prevent apoptosis. As the endonuclease appears to function in the effector stage of apoptosis, the ability of zinc to protect against apoptosis would likely depend on the inhibition of an upstream effect. Indeed, others have shown that while zinc can prevent DNA fragmentation, it cannot prevent the morphological changes associated with apoptosis in some systems[51,321,327,328].

3.2.2 Recent Evidence for Zinc-Induced Inhibition of Apoptosis

Recently, several groups have reported that zinc may inhibit apoptosis by interfering with cellular events other than the activation of the Ca^{2+}/Mg^{2+}-sensitive endonuclease. Briefly, low and more physiological doses of zinc have been shown to directly inhibit the activity of caspases[59,331] and to prevent

other upstream events involved in the induction of apoptosis. Wolf et al[59] have demonstrated that low doses of zinc can directly inhibit the activity of caspase-3. Caspase-3 is a protease that displays significant homology with ced-3, and is activated during apoptosis induced by various means. The authors suggest that zinc may inhibit the activity of caspase-3 by coordinating with cysteine or histidine molecules required for the catalytic activity of caspase-3. Others have shown that zinc can inhibit cellular changes associated with apoptosis that occur prior to the cleavage of caspases and the activation of the endonuclease. The dephosphorylation of the retinoblastoma susceptibility protein, an upstream event in apoptosis, was found to be inhibited by low concentrations of zinc[59]. It was suggested that zinc might interfere with the kinase and/or phosphatase activities involved in this step. In addition, zinc has also been shown to prevent the binding of glucocorticoids to glucocorticoid receptors involved in the signal transduction of apoptosis, presumably by interfering with vicinal thiols located in the binding regions of the receptors[321]. Thus, low and more physiological doses of zinc have been shown to inhibit apoptosis, suggesting that zinc may modulate apoptosis independently of inhibition of the endonuclease[59,321,331].

As shown above, the ability of zinc to inhibit apoptosis has been well established and recent results suggest that zinc may modulate apoptosis independently of the inhibition of the endonuclease. In view of zinc's ability to affect many cellular events, including transcription factor binding activity, zinc may also function to prevent apoptosis by altering transcription factor binding activity.

CENTRAL HYPOTHESIS FOR PRESENT STUDIES

In view of the above data, the present studies were predicated on the hypothesis that zinc may regulate apoptosis by modulating transcription factor binding to DNA.

PROJECT AIMS:

While the actual mechanisms regulating the apoptotic pathway have yet to be fully elucidated, regulation appears to depend on the balance between apoptosis-suppressing and apoptosis-inducing factors within the cell. Accordingly, apoptosis can be modulated by transcription factors that regulate these apoptotic factors. This, in turn, suggests that factors regulating transcription factor binding activity can be expected to modulate apoptosis. Of particular interest is the ability of zinc to modulate apoptosis. Recently, zinc has been shown to inhibit the progression of the apoptotic pathway at several points upstream of the endonuclease. Indeed, zinc is involved in numerous aspects of cellular function including transcription factor binding activity. Thus, zinc may also function to prevent apoptosis by altering transcription factor binding activity. In the present studies, the ability of zinc to protect against apoptosis was explored and correlated with changes in transcription factor binding activity. Focus was placed on the transcription factors NFκB and AP-1 in view of their apparent involvement in the regulation of apoptosis.

The objectives of this study were to:

a) Induce apoptosis in cultured human umbilical vein endothelial cells and correlate the extent of apoptosis with changes in transcription factor binding activity. The induction of apoptosis following treatment with IR, TNFα or TPEN was assessed by the characteristic morphological features of apoptotic nuclei using the Hoechst 33258 stain and was confirmed by the typical DNA "laddering"

pattern. The binding activities of the transcription factors NFκB, AP-1 and Sp1 were assessed using the electrophoretic mobility shift assay (EMSA) following experimental treatment.

b) Inhibit IR-induced apoptosis by treatment with zinc pyrithione, a zinc ionophore, and correlate with changes in transcription factor binding activity.

c) Alter NFκB activation or binding activity and correlate with changes in apoptotic behaviour.

METHODS

Reagents

Unless otherwise indicated, all reagents were obtained from Sigma or BDH.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (San Diego, California, USA) and used from passages 2-4. Cells were cultured on gelatin-coated culture dishes in Endothelial Basal Medium (Clonetics) supplemented with 10ng/ml human recombinant epidermal growth factor, 1.0μg/ml hydrocortisone, 50μg/ml gentamicin, 50ng/ml amphotericin B, 12μg/ml bovine brain extract and 2%v/v fetal bovine serum, in a humidified chamber at 37°C and 5% CO_2. To maintain cell populations, proliferating HUVEC were passaged at 80-90% confluency. Briefly, the cells were washed with Hepes buffered saline solution and covered with 0.025% trypsin/ 0.01% EDTA to allow for cell detachment. Trypsin activity was then neutralized with Trypsin Neutralizing Solution (Clonetics). The cells were collected by centrifugation at 200g for 5 minutes and counted using a hemacytometer. The cells were then seeded at a density of 2500 cells per cm^2 and culture medium were changed every 48 hours.

Experimental Treatments

HUVEC were grown to confluency, and then given an additional 24 hours to achieve quiescence prior to experimental treatment. The following treatments were performed:

Radiation: The cells were washed twice with 37°C D-PBS and then irradiated in fresh media. Irradiated cells received a dose of 1000 Rads of gamma-irradiation from a ^{137}Cesium source (Gamma Cell-40, Nordion) at a dose rate of 101.06 Rads per minute. The cells were then incubated for 2 hours (cytosolic and nuclear protein extraction) or 8 hours (Hoechst 33258 staining and DNA electrophoresis).

TNFα, TPEN, CH and Act D: The cells were washed twice with 37°C D-PBS and then incubated in medium containing the appropriate reagent(s). Unless otherwise indicated, all reagent stock solutions were prepared in DMSO. TNFα (20ng/ml, from a stock of 10μg/ml prepared in phosphate buffered saline (PBS)-1%bovine serum albumin), TPEN (10μM, from a stock of 15mM), CH (3μg/ml, from a stock of 10mg/ml) or Act D (0.1μg/ml, from a stock of 10mg/ml), were added directly to fresh media. Control cells received fresh medium alone. The cells were then incubated for 2 hours (cytosolic and nuclear protein extraction) or 8 hours (Hoechst 33258 staining and DNA electrophoresis).

Zinc pyrithione: Cells receiving this additional treatment were processed as follows: The medium was removed and the cells were washed twice with D-PBS. The cells were then incubated in D-PBS containing zinc pyrithione (5μM, from a stock of 50mM prepared in DMSO) for 5 min. The cells were then washed twice with D-PBS, and fresh medium was added. Irradiated cells were treated with zinc pyrithione immediately post-irradiation, while TNFα treated cells were treated immediately prior to the addition of TNFα containing media. The cells were then incubated for 2 hours (cytosolic and nuclear protein extraction) or 8 hours (Hoechst 33258 staining and DNA electrophoresis). Control cells were treated with sodium pyrithione (5μM, from a stock of 50mM prepared in DMSO) or DMSO (0.01%) for 5 minutes. All washes and treatments were done at 37°C.

Hoechst 33258 Staining

Cells were grown on round, gelatin coated (0.5% gelatin and 0.05% chromium potassium), 12mm glass coverslips, and following treatment, were fixed with 0.5ml of 1% glutaraldehyde in PBS for 10 minutes at room temperature (RT). The cells were then washed twice with PBS for 5 minutes, and permeabilized with 0.5ml of 1:1 methanol/acetone for 10 minutes at RT, followed by two five minute PBS washes. The cells were then incubated with Hoechst 33258 (bis-benzimide, 0.05μg/ml in H_2O), a fluorescent DNA binding dye, for 30 minutes at room temperature, protected from light. The excess dye was removed by three PBS

washes, and the coverslips were covered with an "anti-fade" solution (1mg/ml p-phenylenediamine, 90% glycerol in PBS). The nuclear morphology of the cells was then visualized under a Zeiss Axiophot fluorescence microscope.

Agarose DNA Gel Electrophoresis

Agarose DNA gel electrophoresis was performed according to previously described protocols[332-334]. Following the experimental treatment, cells were scraped into ice cold PBS and centrifuged at 200g to pellet the cells. Cells ($5.0X10^6$) were then lysed in 1.25ml of a buffer containing 10mM Tris-HCl (pH 8.0), 10mM ethylenediaminetetra-acetic acid disodium salt (EDTA), 75mM sodium chloride (NaCl) and 0.5% sodium dodecyl sulfate (SDS) for 15 minutes at RT and then centrifuged for 15 minutes at 13000g. The supernatant containing the low molecular weight DNA was treated with proteinase K (100µg/ml) at 50°C for 30 min. The DNA was allowed to precipitate overnight in 60% ethanol and 0.5M NaCl at -20°C. The precipitated DNA was then pelleted by centrifugation for 15 minutes at 13000g at 4°C and resuspended in 100µl of Tris-EDTA buffer (TE, containing 10mM Tris-HCl and 1mM EDTA). A phenol-chloroform extraction was performed and excess phenol was removed with a subsequent chloroform wash. The DNA was again precipitated for a minimum of one hour in 60% ethanol and 0.5M NaCl at -20°C. The precipitated DNA was resuspended in TE buffer and incubated for 30 minutes with 100ug/ml of RNase A at 37°C. U.V. absorbance at 260 nm was used to determine the concentration of DNA, while the ratio of 260nm to 280nm was used to assess the purity of the DNA. Ten µg of DNA were subjected to electrophoresis on a 1.5% agarose gel in TAE buffer (400mM Tris, 300mM Acetic Acid and 20mM EDTA, pH=8.0) at 100 Volts for 50 minutes. The gel was then stained for thirty minutes at room temperature with 0.5µg/ml ethidium bromide and the DNA bands were visualized with U.V. light.

Preparation of Cytosolic and Nuclear Extracts[255,335,336]

Cells were grown on 100mm^2 culture dishes, and following treatment, were scraped into ice cold PBS and collected by centrifugation at 200g for 5 minutes. The cells were then resuspended and washed once in 1 ml of ice cold PBS and centrifuged at 200g for 5 minutes at 4°C. The cells were resuspended in 1 ml of Buffer A (10mM Hepes, 10mM KCl, 1.5mM MgCl2, pH=7.9, 1.5mM DTT and 0.5mM phenyl methyl sulphonyl fluoride (PMSF)) and centrifuged at 200g for 5 minutes at 4°C. The cells were then resuspended and lysed in 300µl of Buffer A containing 0.1% Nonidet P-40 for 25 minutes on ice. The homogenate was then spun at 20 000g for 10 minutes at 4°C. The supernatent containing cytosolic proteins was combined with an equal volume of Buffer C (20mM Hepes, 50mM KCl, 1.0 mM EDTA, 0.1mM EGTA, 20% glycerol, pH=7.9, 0.5mM DTT and 0.5mM PMSF) and was stored at -80°C. The pelleted nuclei were washed by resuspension in 1ml of Buffer A and spun at 20 000g for 1 minute. The supernatent containing residual cytosolic proteins was discarded and the pelleted nuclei were resuspended in 35µl of Buffer B (20mM Hepes, 420mM NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 25% glycerol, pH 7.9, 0.5mM DTT, 0.5mM PMSF, and the protease inhibitors spermidine, spermine, aprotinin, leupeptin and pepstatin) for 45 minutes on ice in order to extract the nuclear proteins. The nuclear extract was then obtained following centrifugation at 20 000g for 15 minutes at 4°C, and was combined with an equal volume of Buffer C and stored at –80°C.

Determination of Protein Concentration

The protein concentration in the nuclear and cytosolic extracts was determined using the Bradford Assay (Biorad) using bovine serum albumin as the standard.

Electrophoretic Mobility Shift Assay (EMSA)[255,336]

Equal amounts of nuclear protein (5μg) were incubated with poly dI-dC (5μg from a stock of 2.5μg/μl in TE buffer) for 10 minutes at RT. This reaction mixture was then incubated with 0.2ng of 5'end-^{32}phosphorus-labelled double stranded oligonucleotide probe for 20 minutes at RT to allow the binding of nuclear proteins with the labeled probe. Loading buffer (5μl of a mixture containing 20mM Hepes, 100mM KCl, 60% glycerol, 0.5mM EDTA, 0.5mM EGTA and 0.125% bromophenol blue) was added to the reaction mixture prior to the electrophoresis on a 5% native polyacrylamide gel. The gels were run in Tris-Glycine solution for 1.5 hours at 200V and were then dried between filter paper and cellophane for 1.5 hours at 80°C under vacuum. The dried gels were exposed to X-ray film (Cronex) for up to 2 days at −80°C. For competition assays, the reaction mixture was incubated with a 125-fold excess of unlabeled probe for 20 minutes at RT prior to the addition of the labeled probe. For supershift assays, the reaction mixture was incubated with 2μg of rabbit polyclonal anti-NFκB p50 or p65 antibody (Santa Cruz Biotechnology) for 20 minutes at RT immediately subsequent to the addition of the labeled probe. The bound antibody retards the mobility of the protein-DNA complex, resulting in a shifted band.

The consensus oligonucleotides for the transcription factors NFκB (5'-ACT TGA GGG GAC TTT CCC AGG C-3'), AP-1 (5'-CGC TTG ATG AGT CAG CCG GAA-3') and Sp1 (5'-ATT CGA TCG GGG CGG GGC GAG C-3') (Promega) and were labeled as suggested by Promega with minor modifications. Briefly, oligonucleotides (20ng), T4 Polynucleotide kinase and [γ^{32}P]ATP (60μCi) were mixed in kinase buffer (50mM Tris-HCl, pH 7.6, 10mM $MgCl_2$, 5% glycerol and 5mM DTT) and incubated at 37°C for 1 hour. Labeled oligonucleotides were removed by centrifugation through a G-25 Sephadex Column at 8500rpm for 20 minutes. The labeled oligonucleotides were then diluted such that 2μl of the probe mixture contained approximately 50000-100000cpm.

Western Blotting

Equal amounts of cytosolic protein (3μg) were diluted 1:1 in sample buffer (0.125M Tris-HCl pH 6.8, 2.6%SDS, 25% glycerol, 0.1ml beta-mercaptoethanol and bromo phenol blue). The mixture was placed in boiling water for 5 minutes to denature the proteins and was then subjected to SDS-PAGE for 2.5 hours at 100V in running buffer. The gels consisted of a stacking gel (4.5% acrylamide, 0.125M Tris-HCl pH=6.8, 0.1% SDS, 0.6% ammonium persulfate and 0.2% TEMED in H_2O) and a 10% running gel (10% acrylamide, 0.3% bis acrylamide, 8% glycerol, 0.375 Tris-HCl pH=8.8, 0.1% SDS, 0.04% ammomium persulfate and 0.05% TEMED in H_2O). After electrophoresis (100V, 50 minutes), the gels were equilibriated for 15 minutes in ice cold transfer buffer (25mM Tris HCl, 20% methanol, and 192mM glycine), then transferred onto a polyvinyllidene difluoride membrane for 1 hour at 100V. The blots were then blocked overnight in 5% skimmed milk in Tris-Buffered Saline containing 0.1% Tween-20 (TBS-T) at 4°C with constant shaking. The blots were then washed with TBS-T and incubated for 1.5 hours in primary antibody (anti-IκBα, Santa Cruz Biotechnology) diluted 1:1000 in 2% skimmed milk in TBS-T and sodium azide at RT with constant shaking. The blots were then washed with TBS-T and incubated for 30 minutes in horseradish peroxidase labeled goat anti-rabbit IgG diluted 1:10000 in 2% skimmed milk in TBS-T at RT with constant shaking. Following treatment with the secondary antibody, the blots were extensively washed with TBS-T and incubated for 1 minute with chemiluminescent substrate. The blots were then exposed to X-ray film for 1-5 minutes.

Statistical Analysis

Data were analyzed using Sigma Plot for Windows software from Jandel Scientific. Values are expressed as mean ± standard deviation. Statistical significance was determined by Students t-test where only P values of less than 0.05 were considered significant.

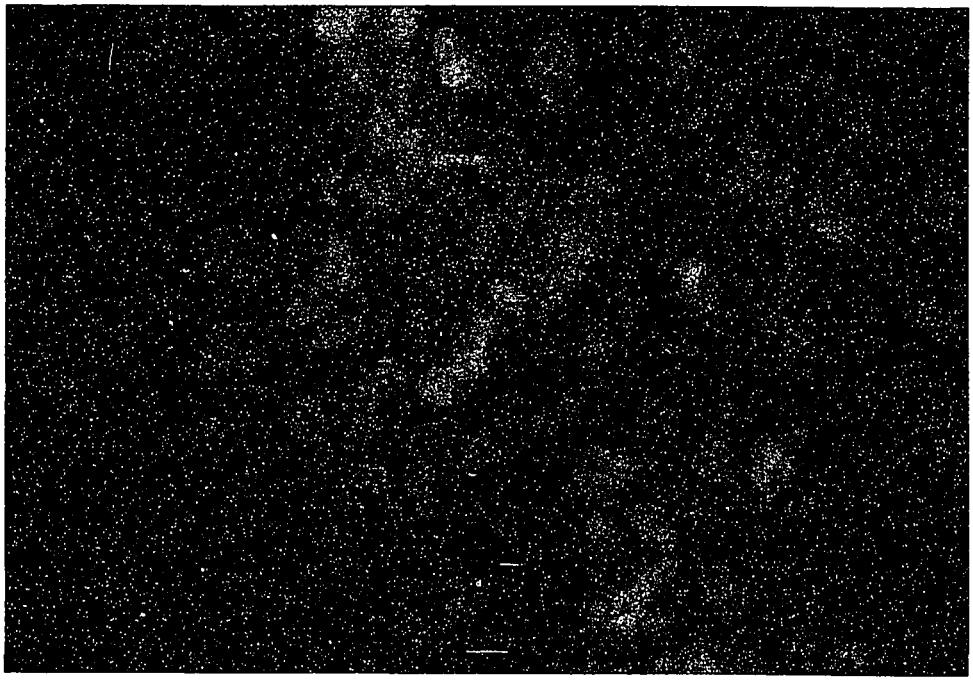
RESULTS

Determination of Apoptosis

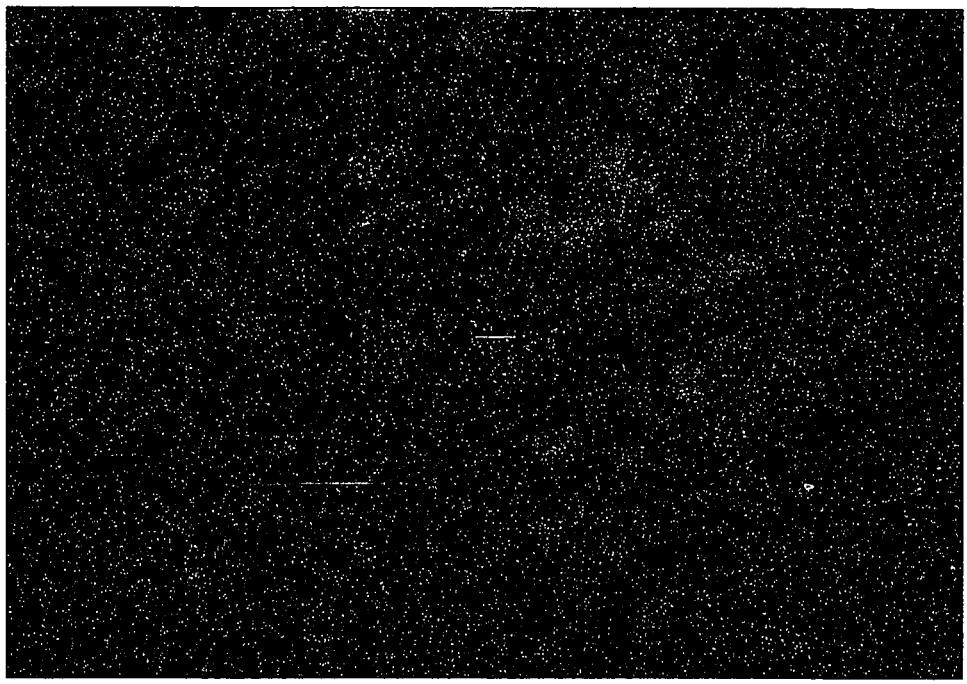
Apoptosis was detected by the characteristic morphological features of the nuclei using the Hoechst 33258 stain. Cells undergoing apoptosis condense and fragment their nuclei in a distinct pycnotic pattern. Using this morphological characteristic of apoptosis, the ability of cells to undergo apoptosis and the extent of the observed apoptosis were determined. Three putative apoptosis-inducing treatments were performed and the percent of the cells undergoing apoptosis was determined after 8 hours (Figures 1and 2). IR (1000 Rads) was found to significantly induce apoptosis (9.7 ± 1.2%) when compared to control cells, which exhibited a basal level of apoptosis (1.7 ± 0.5%). Incubation with the heavy metal chelator TPEN (10μM) was also found to induce a significant amount of apoptotic morphology (13.9 ± 1.3%). In HUVEC, TNFα has been reported to be able to either induce apoptosis, or to require the presence of another agent for a pro-apoptotic effect. Here, exposure of HUVEC to 20ng/ml of TNFα for 8 hours did not result in a significant increase in apoptotic morphology (1.7 ± 0.2%). The lack of a pro-apoptotic effect was not a result of a lack of biological activity of the TNFα used since the treatment of bovine aortic endothelial cells with TNFα under the same conditions induced significant apoptosis (not shown). Further confirmation of apoptotic cell death was obtained by agarose DNA gel electrophoresis of low molecular weight DNA. The activation of an endonuclease that cleaves DNA into multiples of 180-200 base pair fragments is a hallmark of apoptotic cell death. When viewed on an agarose electrophoretic gel, these fragments appear as 'ladders'. Treatment of cells with 0.1μg/ml Act D for 8 hours induced significant apoptotic morphology (13.4 ± 1.7%) as viewed with the Hoechst 33258 stain (Figure 1). Cells treated with Act D for 8 hours and then harvested for low molecular weight DNA extraction and agarose gel electrophoresis exhibited the typical DNA 'laddering' pattern characteristic of apoptosis (Figure 3), thus confirming the identification of morphologically distinct nuclei using the Hoechst 33258 stain as apoptotic.

Figure 1. Morphological identification of apoptotic nuclei by Hoechst 33258. Cells were stained with Hoechst 33258 and examined for apoptotic nuclear morphology following 8 hours of experimental treatment. Panel A shows control cells, with little evidence of apoptotic morphology. Nuclei exhibiting apoptotic morphology were also absent in cells treated with TNFα (20ng/ml, Panel B). Nuclei exhibiting apoptotic morphology are present in IR- (1000 Rads), TPEN- (10μM) and Act D- (0.1μg/ml) treated cells (Panels C, D and E, respectively). Nuclei representative of apoptotic cells are indicated by arrows. (figures are representative of at least 4 independent trials)

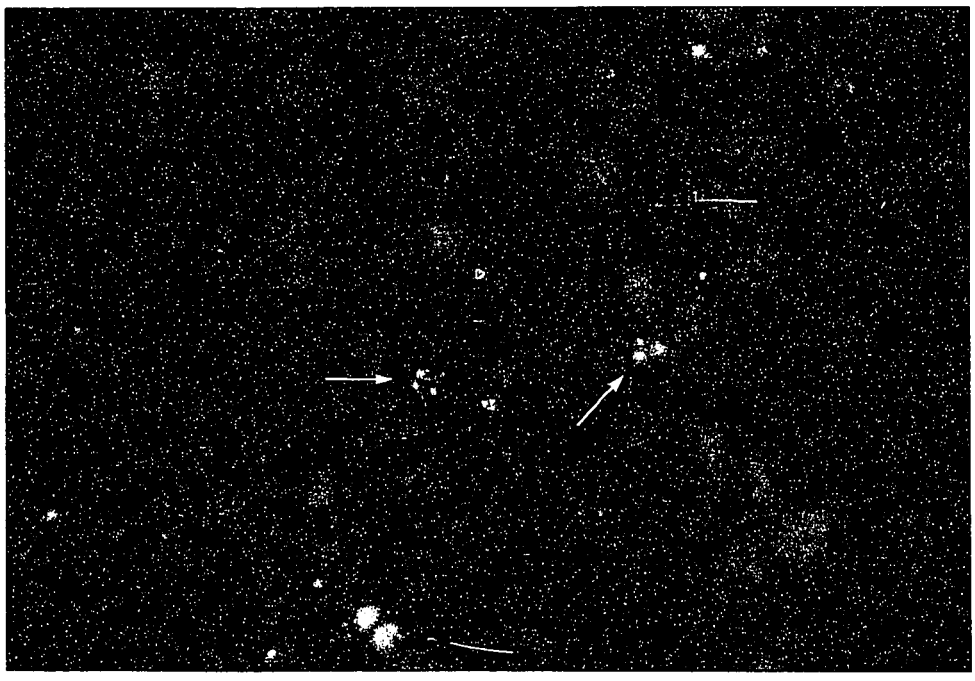
A



B



C



D

E

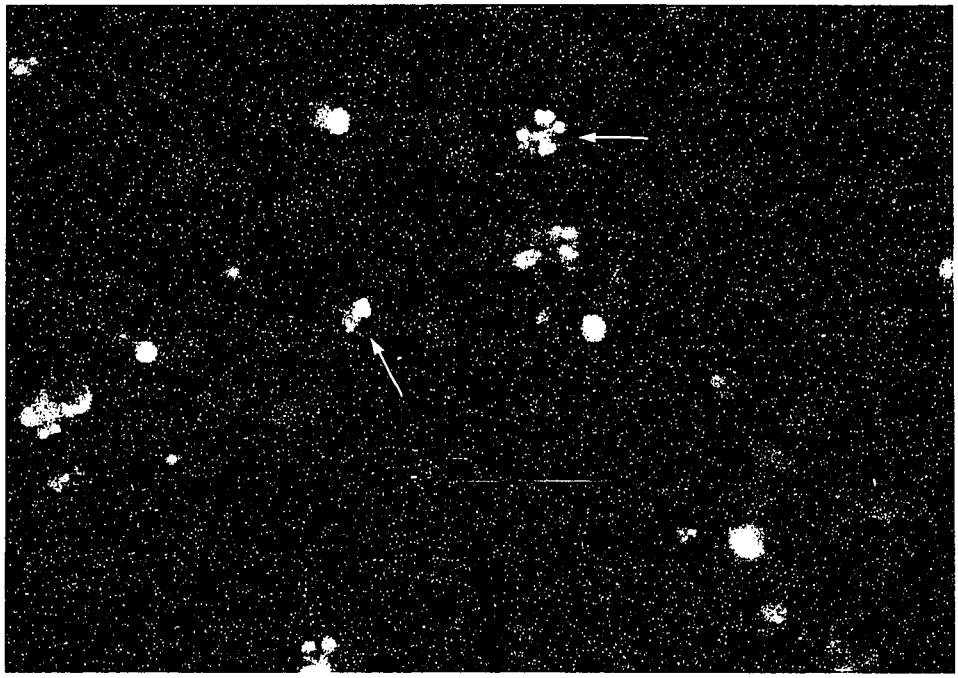


Figure 2. Quantification of percent apoptotic cells. The percent of cells with apoptotic morphology was assessed in Hoechst 33258-stained coverslips by determining the percent of nuclei displaying apoptotic morphology after 8 hours of treatment. The number of nuclei exhibiting apoptotic morphology from ten fields of view per coverslip were counted and expressed as a percent of the total number of nuclei. IR (1000 Rads) induced a significant increase in apoptotic morphology as did treatment with TPEN (10μM) over control cells. In contrast, no evidence of apoptotic morphology was evident in cells treated with TNFα (20ng/ml). Incubation with Act D (0.1μg/ml) significantly increased the occurrence of apoptotic nuclei. The carrier, DMSO, had no effect on apoptotic cell death. (* $P<0.05$ versus control, n=4)

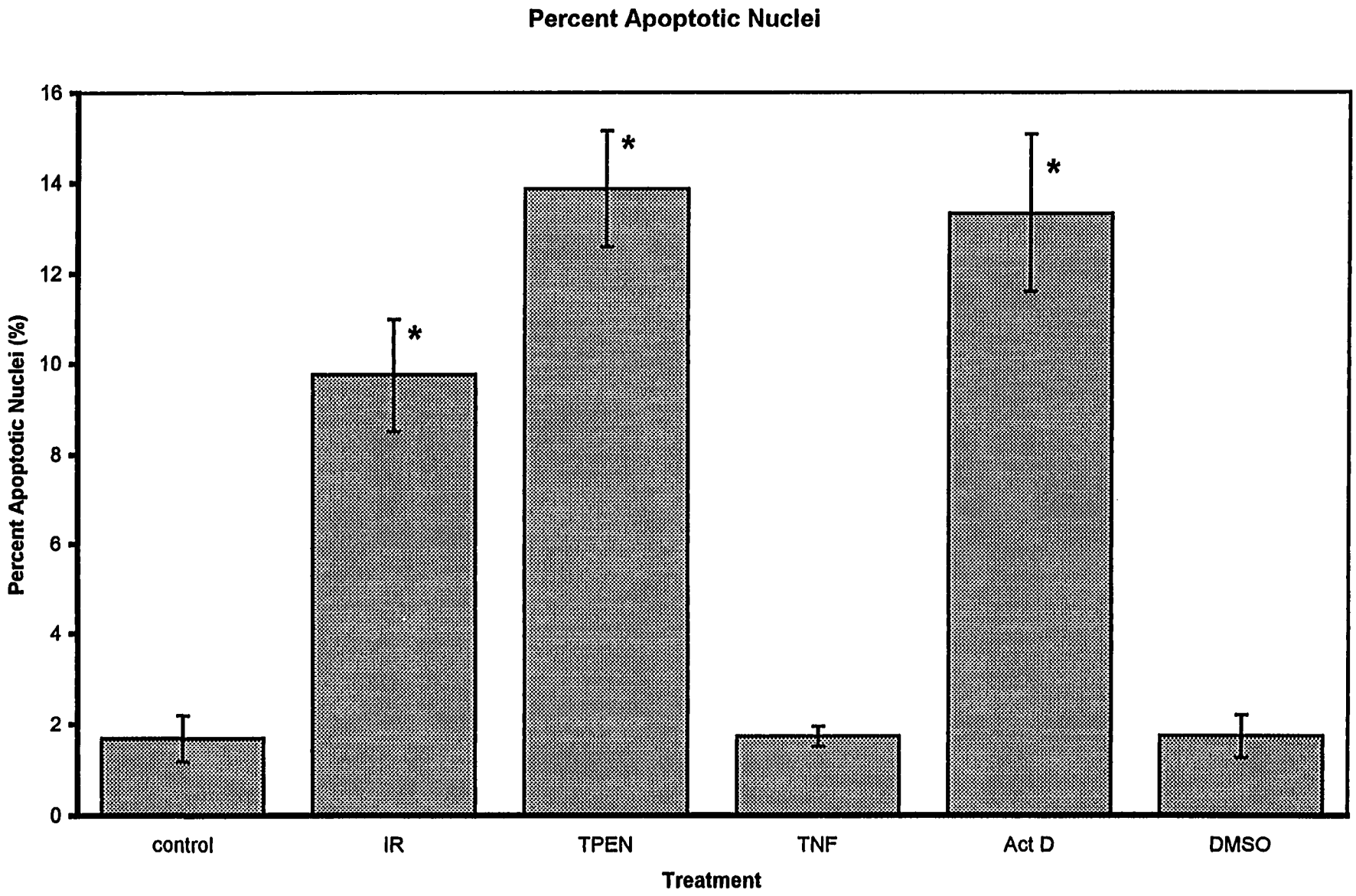
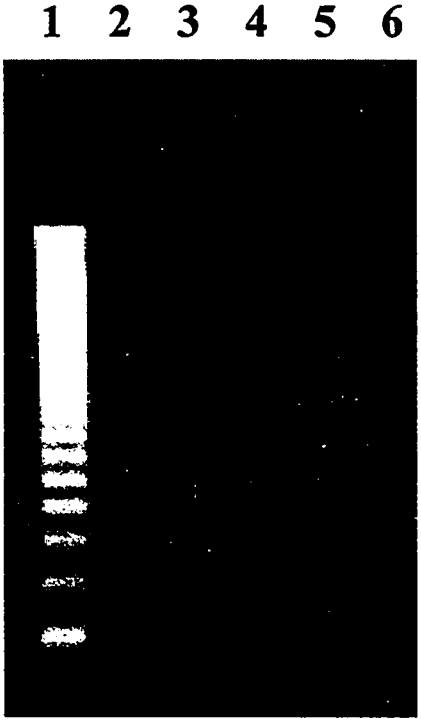

Percent Apoptotic Nuclei
Percent Apoptotic Nuclei (%)
16
14
12
10
8
6
4
2
0
*
*
*
control
IR
TPEN
TNF
Act D
DMSO
Treatment

Figure 3. Low molecular weight DNA fragmentation. The pattern of low molecular weight DNA fragmentation obtained from control cells (Lanes 2-4) or cells treated with Act D (0.1μg/ml) for 8 hours (Lanes 5 and 6) was assessed and compared to that obtained with irradiated thymocytes (Lane 1), a standard for DNA laddering. (figure is representative of three trials)


1 2 3 4 5 6

Transcription Factor Binding Activity

To correlate changes in transcription factor binding activity with treatments that induce apoptosis, nuclear extracts were obtained from cells after two hours of treatment and the binding to ^{32}P-labelled concensus sequences of the transcription factors NFκB, AP-1 and Sp1 by EMSA was measured (Figure 4). Of particular interest was the NFκB binding activity in view of the increasingly apparent role of NFκB in apoptosis. While both IR (1000R) and TPEN (10μM) induced apoptosis in HUVEC, only IR was found to induce a significant increase in NFκB binding activity (170.2 ± 14.0%) over control levels (Figure 5). Nuclear extracts from cells treated with TPEN showed no increase in NFκB binding activity (110.8 ± 6.6%). Treatment of cells with 20ng/ml TNFα, which, as shown above, did not induce an apoptotic response, nevertheless dramatically increased NFκB binding activity by 4.5-fold (433.4 ± 44.4%) over control levels.

Nuclear extracts from cells treated with IR, TPEN or TNFα were also probed for binding activity to the consensus sequences for the transcription factors AP-1 or Sp1 (Figure 4). The DNA binding activities of the transcription factors AP-1 and Sp1 were unaltered by any of these treatments, with the exception of TPEN, which induced a significant decrease in Sp1 binding activity (Figure 5).

The specificity of the observed banding patterns for all three probes was confirmed by competition experiments (Figure 6). Supershift analysis of the specific NFκB band indicated a heterodimer composed of the p50 and the p65 subunits of the NFκB family.

Figure 4. Determination of transcription factor binding activity. The binding activity of nuclear extracts to ^{32}P-labelled concensus sequences for the transcription factors NFκB, AP-1 and Sp1 were analyzed by EMSA. The nuclear extracts were prepared following two hours of experimental treatment, and equal amounts of nuclear protein (5μg) were assayed for binding activity. NFκB binding activity: IR (1000 Rads) and treatment with TNFα (20ng/ml) showed an increase in NFκB binding activity over control levels, while cells treated with TPEN (10μM) displayed no change in NFκB binding activity. AP-1 binding activity: The binding activity of AP-1 was unaltered by the above treatments. Sp1 binding activity: Treatment with TPEN decreased basal Sp1 binding activity, while IR or TNFα had no effect. (this figure is representative of at least 3 independent trials)

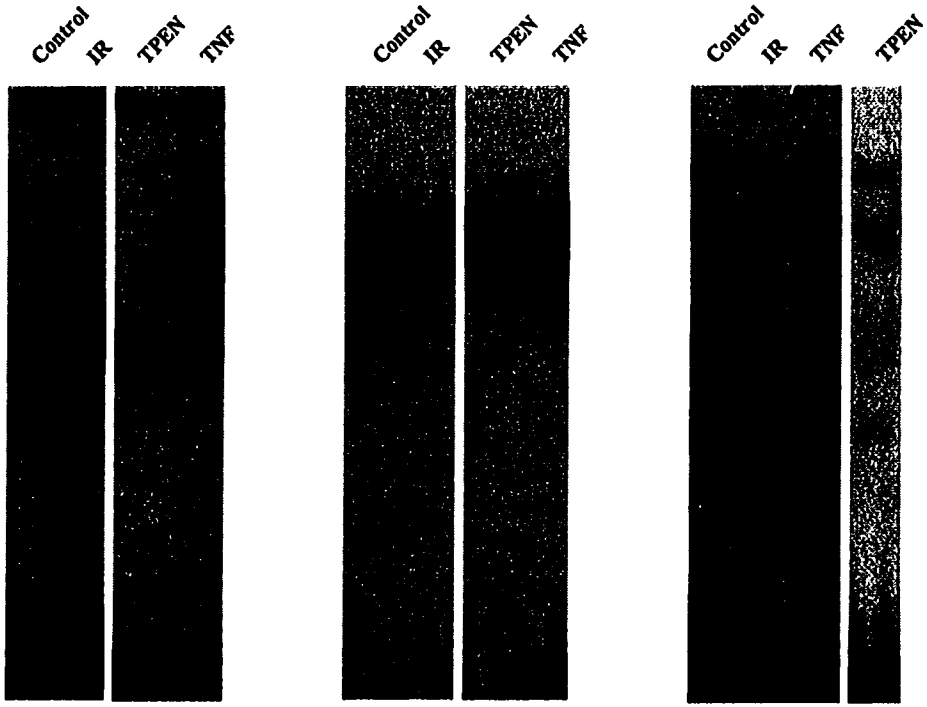

NFkappa B
AP-1
Sp1
Control
IR
TPEN
TNF
Control
IR
TPEN
TNF
Control
IR
TNF
TPEN

Figure 5. Quantification of transcription factor binding activity. The transcription factor binding activity was assessed by EMSA following two hours of experimental treatment and was analyzed by quantitative densitometry. The values are expressed as a percentage of control levels. NFκB binding activity in nuclear extracts from irradiated cells (IR, 1000 Rads) was significantly elevated, while cells treated with TPEN (10μM) displayed no change in NFκB binding activity. NFκB binding activity was increased 4.5-fold in cells treated with TNFα (20ng/ml). No significant changes in AP-1 binding activity were seen with any of the treatments, and only TPEN induced a significant decrease in Sp1 binding activity, as compared to control values. (*$P<0.05$ versus control groups, the n value for each treatment is as follows: control n=6, IR n=6, TPEN n=3, TNFα n=5)

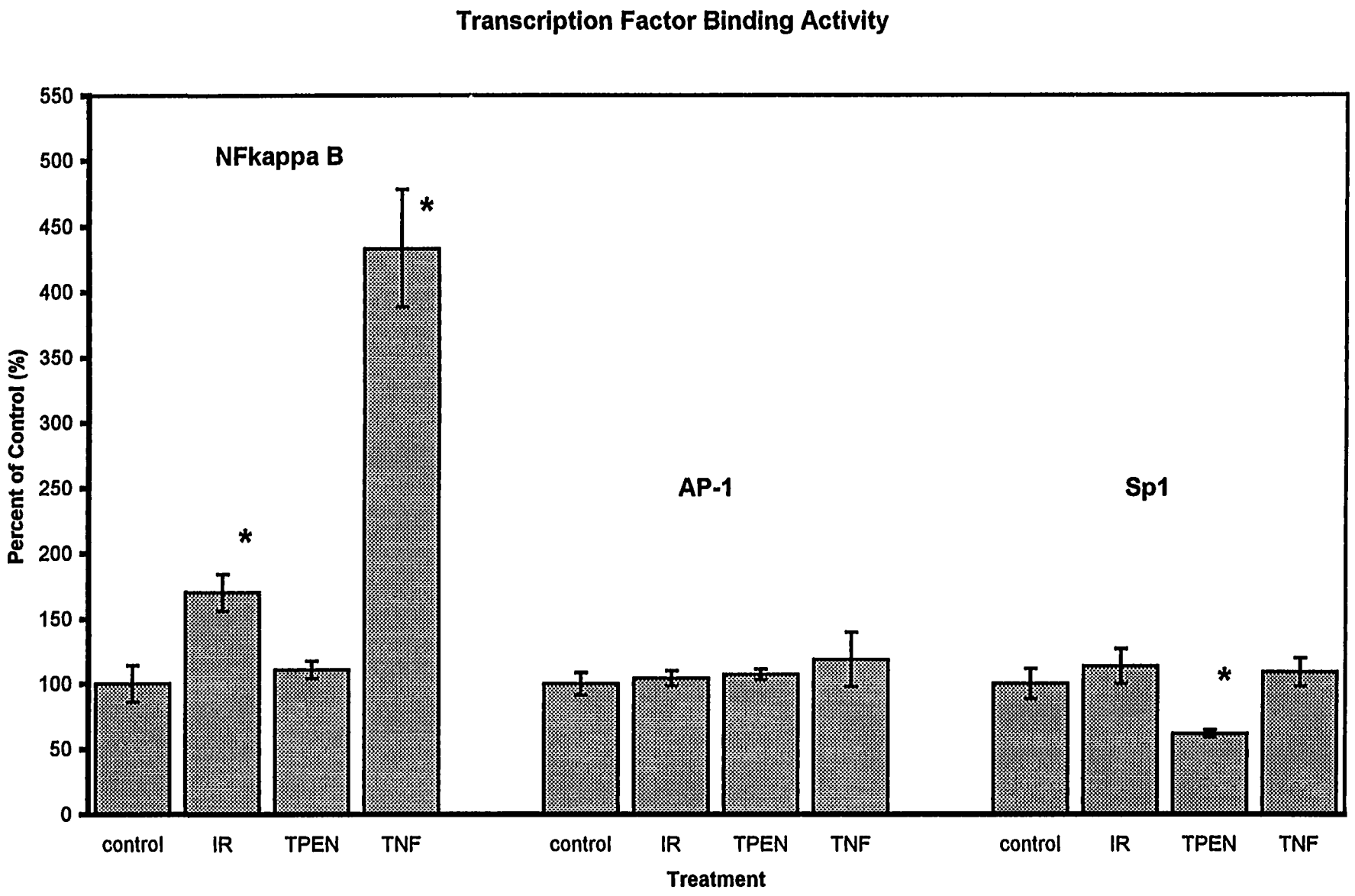
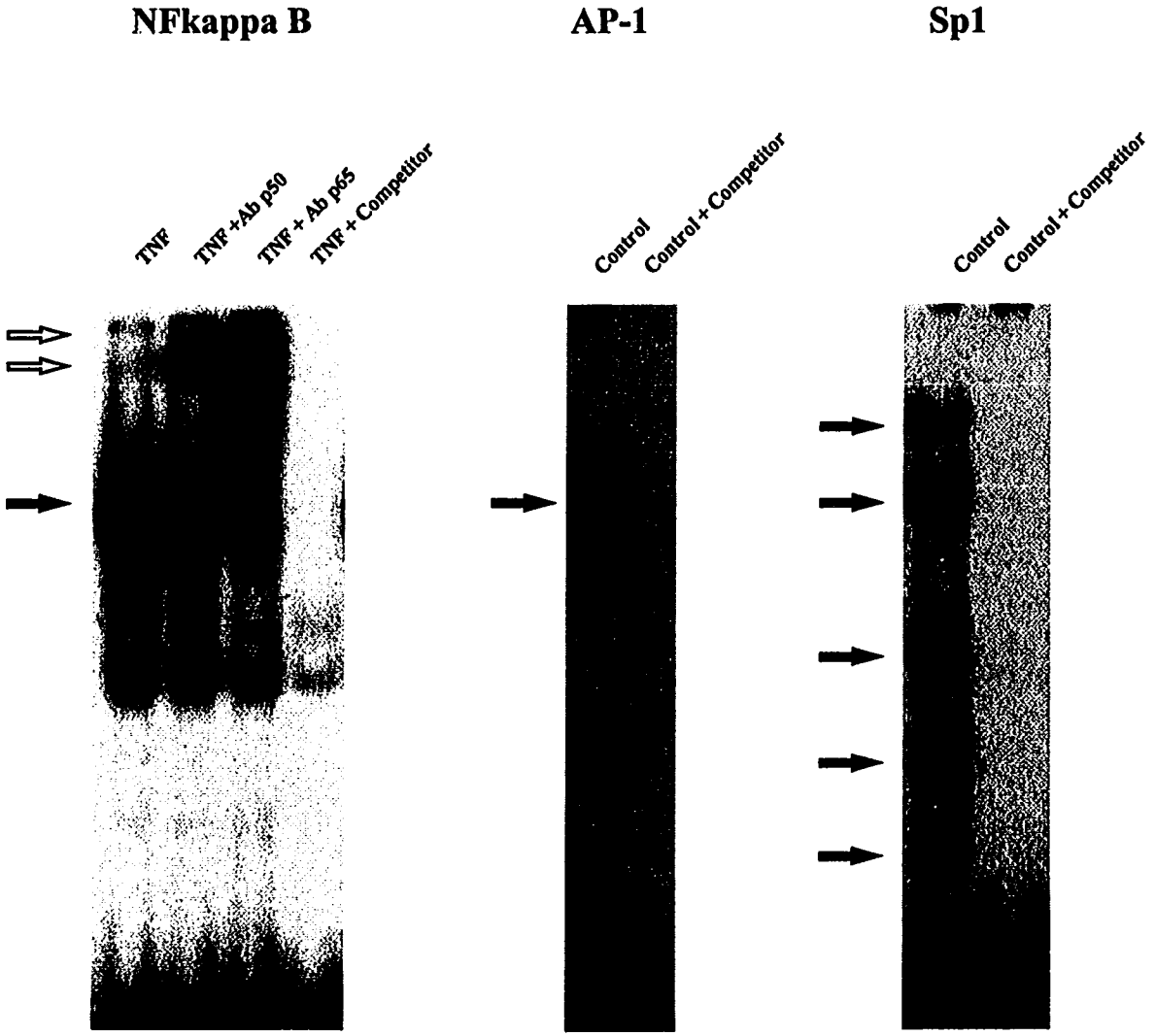

Transcription Factor Binding Activity
NFkappa B
AP-1
Sp1
Percent of Control (%)
550
500
450
400
350
300
250
200
150
100
50
0
*
*
*
control
IR
TPEN
TNF
control
IR
TPEN
TNF
control
IR
TPEN
TNF
Treatment

Figure 6. Specificity of nuclear binding activities. The binding activity of nuclear extracts to ^{32}P-labelled concensus sequences for the transcription factors NFκB, AP-1 and Sp1 was analyzed by EMSA. The nuclear extracts were prepared following two hours of experimental treatment, and equal amounts of nuclear protein (5μg) were assayed for binding activity. Competition experiments were performed to determine the specificity of the observed banding patterns (outlined in the EMSA section of Methods). NFκB binding activity: Cells treated with TNFα (20ng/ml) displayed significant NFκB binding activity. Treatment of this nuclear extract with a 125-fold excess of unlabelled NFκB probe abolished the specific banding pattern. Incubation of the nuclear extract with antibodies to either the p50 or the p65 subunit of NFκB resulted in a supershift of the specific NFκB band. AP-1 binding activity: Incubation of the nuclear extracts from control cells with a 125-fold excess of unlabelled AP-1 probe identified the banding pattern as specific. Sp1 binding activity: Incubation of the nuclear extracts from control cells with a 125-fold excess of unlabelled Sp1 probe identified the specific banding patterns. (arrows indicate the specific banding patterns for all three probes, empty arrows indicate the supershifted p50 and p65 NFκB subunits)


NFkappa B
AP-1
Sp1
TNF
TNF +Ab p50
TNF + Ab p65
TNF + Competitor
Control
Control + Competitor
Control
Control + Competitor

Zinc Inhibits Apoptosis and NFκB Binding Activity

As described above, zinc has long been implicated in the protection of cells against apoptosis in irradiated cells, and recent evidence suggests that zinc may function at numerous locations in the cell to exert its protective effect. We sought to determine if this protection against apoptosis is associated with changes in transcription factor binding activity. HUVEC were treated immediately post-IR with zinc pyrithione, a zinc ionophore that rapidly increases intracellular zinc levels. A 5 minute treatment with 5 μM zinc pyrithione significantly decreased apoptosis in irradiated cells (4.2 ± 0.9%) compared to cells receiving IR alone (9.7 ± 1.2%, Figure 7). This effect was specific for zinc since neither sodium pyrithione (9.4 ± 1.7%) nor the carrier DMSO (9.5 ± 0.4%) significantly altered the IR-induced apoptosis (Figure 7).

As shown in Figure 8, IR-induced apoptosis was associated with an increase in NFκB binding activity (170.2 ± 14.0%, Figure 9). However, zinc pyrithione treatment immediately post-irradiation significantly decreased the NFκB binding activity (103.2 ± 9.8%) to control levels (Figure 9). This effect was specific for zinc as neither sodium pyrithione (173.7 ± 9.4%) nor the carrier DMSO (178.8 ± 14.3%) significantly altered the IR-induced increase in nuclear NFκB binding activity. Zinc pyrithione alone (99.6 ± 13.2%) had no effect on NFκB binding activity.

While the nuclear binding activity of the transcription factor AP-1 (Figure 8) was not altered by IR (104.2 ± 5.8%), treatment of the cells with 5μM zinc pyrithione for 5 minutes alone (48.9 ± 12.3%) or immediately post-IR (64.4 ± 8.5%) significantly decreased AP-1 binding activity to below control levels (Figure 9). This effect appears to be specific for zinc as treatment with sodium pyrithione (108.7 ± 5.8%) or DMSO (105.7 ± 3.8%) immediately post-IR did not alter the control levels of AP-1 binding activity. No significant changes in Sp1 binding activity were found with any of the treatments (Figures 8 and 9). Thus, treatment of cells with zinc pyrithione protected against the IR-induced apoptosis and inhibited the IR-induced NFκB binding activity. Treatment with zinc pyrithione also inhibited the basal AP-1 binding activity in irradiated and non-irradiated cells.

Figure 7. Zinc pyrithione-induced protection against apoptosis. The percent of cells with apoptotic morphology was assessed in Hoechst 33258-stained coverslips by determining the percent of nuclei displaying apoptotic morphology after 8 hours of treatment. The number of nuclei exhibiting apoptotic morphology from ten fields of view per coverslip were counted and expressed as a percent of the total number of nuclei. Zinc pyrithione treatment (ZnPyr, 5μM for 5 minutes) immediately post-IR (1000 Rads) significantly reduced the occurrence of apoptotic nuclei as compared to IR alone. Treatment with sodium pyrithione (NaPyr) or the carrier DMSO had no effect on the IR-induced apoptosis. Zinc pyrithione, sodium pyrithione or DMSO treatments alone showed no significant differences in apoptotic morphology as compared to control. (*$P<0.05$ versus control, °$P<0.05$ versus irradiated cells, n=4)

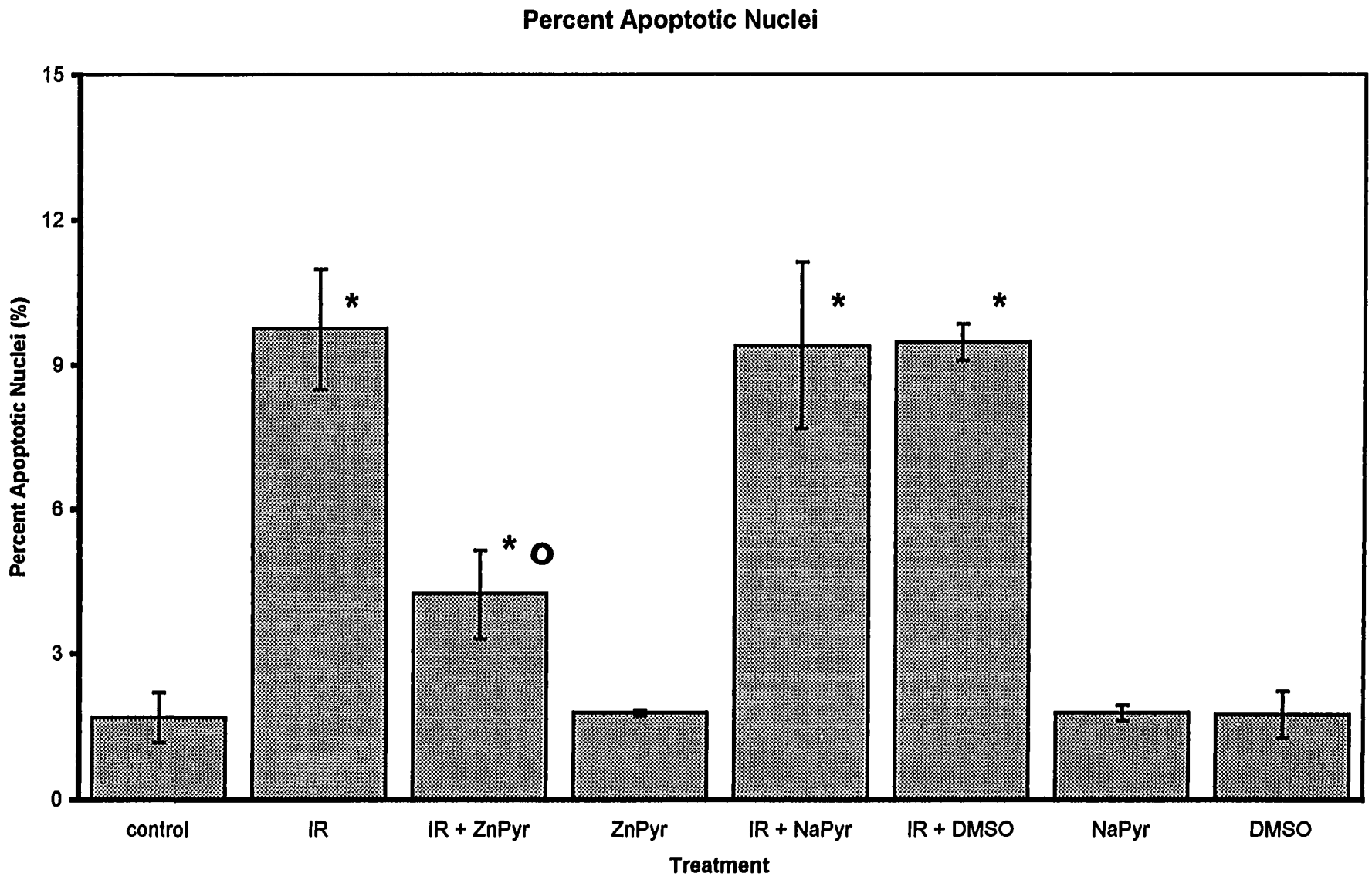

Percent Apoptotic Nuclei
Percent Apoptotic Nuclei (%)
15
12
9
6
3
0
*
*
* o
*
*
control
IR
IR + ZnPyr
ZnPyr
IR + NaPyr
IR + DMSO
NaPyr
DMSO
Treatment

Figure 8. Transcription factor activity in response to irradiation and zinc pyrithione. The binding activity of nuclear extracts to ^{32}P-labelled concensus sequences for the transcription factors NFκB, AP-1 and Sp1 were analyzed by EMSA. The nuclear extracts were prepared following two hours of experimental treatment, and equal amounts of nuclear protein (5μg) were assayed for binding activity. NFκB binding activity: IR (1000 Rads) induced an increase in NFκB binding activity, which was abolished by treatment with 5μM zinc pyrithione (ZnPyr) for 5 minutes immediately post-IR. Treatment with sodium pyrithione (NaPyr) or DMSO immediately following IR did not affect the IR-induced increase in NFκB binding activity. Zinc pyrithione treatment alone had no effect on NFκB binding activity. AP-1 binding activity: The binding activity of AP-1 was not altered by IR. However, treatment with zinc pyrithione (5μM for 5 minutes) alone or post-IR decreased the basal levels of AP-1 binding activity. This effect was not seen by treatment with sodium pyrithione or DMSO post-IR. Sp1 binding activity: The binding activity of Sp1 was unaltered by all treatments. (figure is representative of at least 3 independent trials)

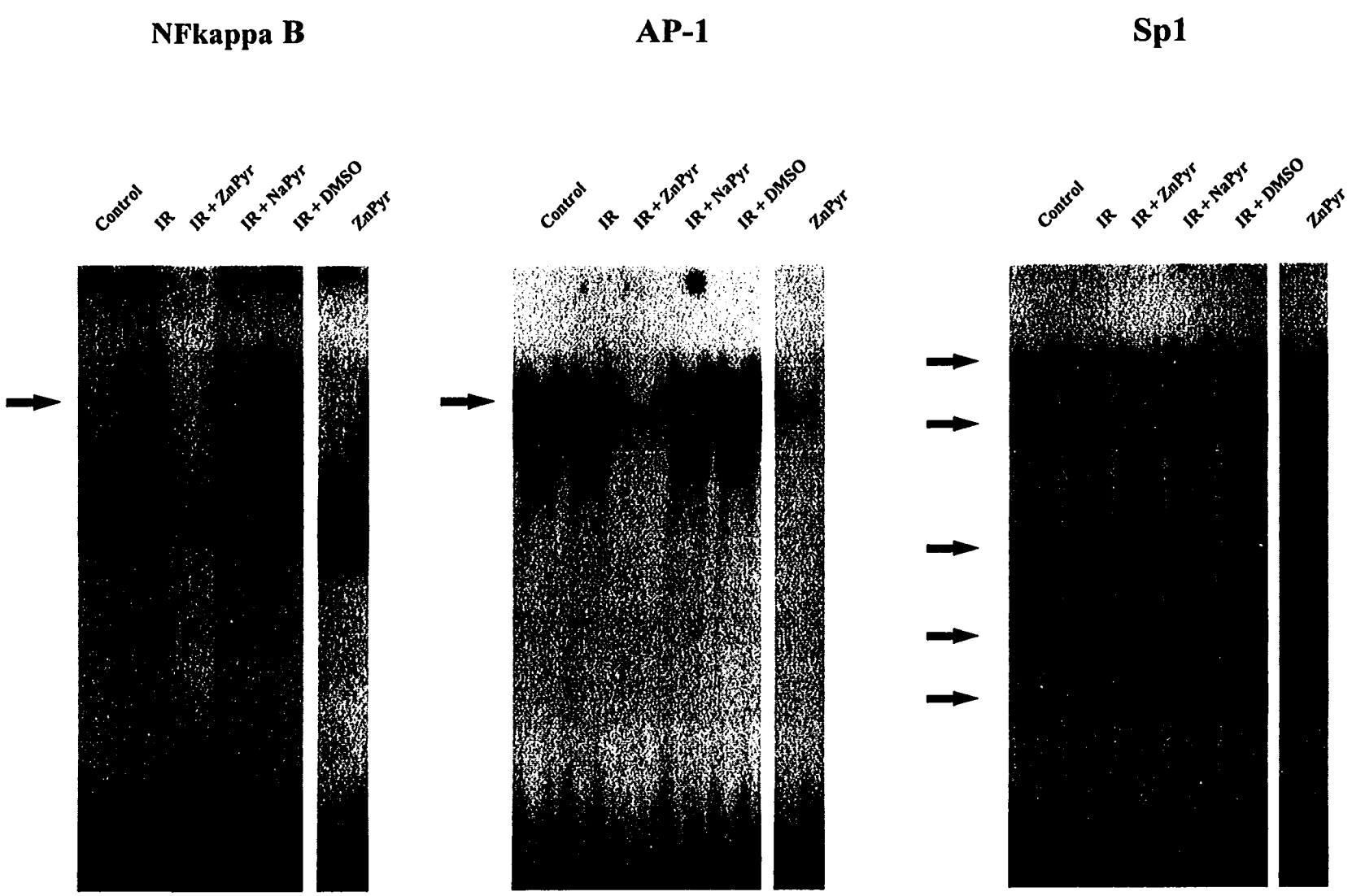
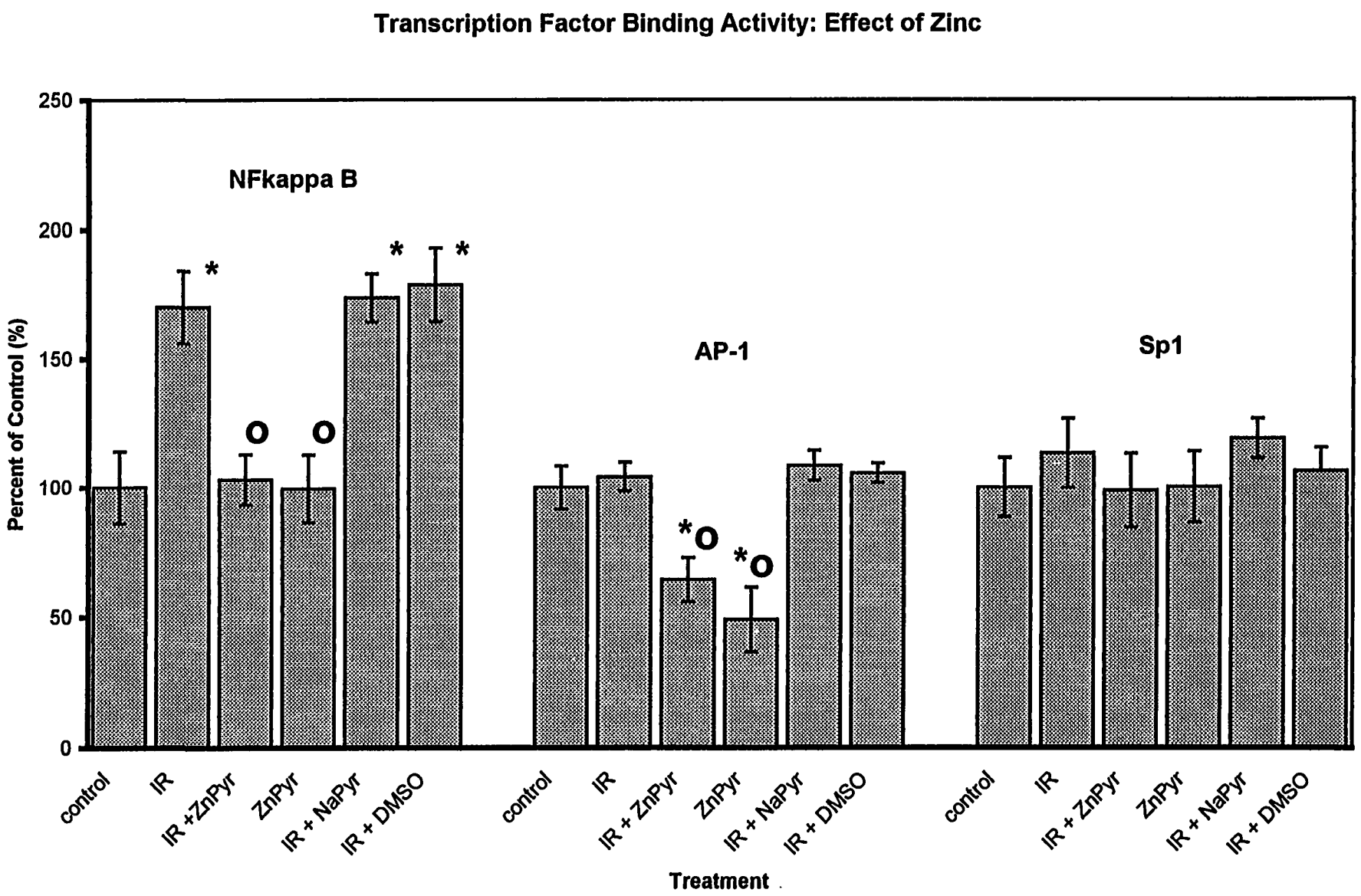

NFkappa B
AP-1
Sp1
Control
IR
IR + ZnPyr
IR + NaPyr
IR + DMSO
ZnPyr

Figure 9. Quantification of transcription factor binding activity in response to irradiation and zinc pyrithione. The transcription factor binding activity was assessed by EMSA following two hours of experimental treatment and was analyzed by quantitative densitometry. The values are expressed as a percentage of control levels. IR (1000 Rads) induced a significant increase in NFκB binding activity over control. Zinc pyrithione (ZnPyr) treatment immediately post-IR significantly reduced nuclear NFκB binding activity. Treatment with sodium pyrithione (NaPyr) or with the carrier DMSO had no effect on the IR-induced increase in nuclear NFκB binding activity. Zinc pyrithione treatment alone had no effect on nuclear NFκB binding activity. The nuclear binding activity of the transcription factor AP-1 was not altered by IR. However, zinc pyrithione treatment (5μM for 5 minutes) alone or immediately post-IR decreased AP-1 binding activity to below control levels. Treatment with sodium pyrithione or DMSO immediately post-IR had no effect on AP-1 binding activity. No significant changes in Sp1 binding activity were found with any of the treatments. (*$P<0.05$ versus control group, °$P<0.05$ versus IR group, the n value for each treatment is as follows: control n=6, IR n=6, IR + ZnPyr n=5, ZnPyr n=3, IR + NaPyr n=3, IR + DMSO n=4)


Transcription Factor Binding Activity: Effect of Zinc
NFkappa B
AP-1
Sp1
Percent of Control (%)
250
200
150
100
50
0
control
IR
IR +ZnPyr
ZnPyr
IR + NaPyr
IR + DMSO
Treatment

Zinc also Inhibits NFκB Binding Activity in TNFα-Treated Cells

In view of the zinc-induced inhibition of IR-induced NFκB binding activity, we sought to determine if zinc could also inhibit NFκB induced by other means (Figures 10 and 11). TNFα (20ng/ml) was found to dramatically increase NFκB binding activity (433.4 ± 44.5%) in HUVEC (Figure 11). However, pretreatment of the cells with 5μM zinc pyrithione for 5 minutes significantly reduced NFκB binding activity (56.9 ± 13.4%) to below control levels. The effect was specific for zinc as neither sodium pyrithione (461.3 ± 5.5%) nor the carrier DMSO (456.4 ± 10.7%) significantly altered the TNFα-induced increase in nuclear NFκB binding activity. Zinc pyrithione treatment alone (99.6 ± 13.2%) had no effect on NFκB binding activity (Figure 5). Thus, it appears zinc may be acting to inhibit NFκB at a point after the convergence of the TNFα and IR-induced signals to activate NFκB, or further downstream, to inhibit the binding of activated NFκB.

As described above, TNFα did not alter the nuclear binding activity of the transcription factor AP-1 (Figure 10). However, treatment of the cells with 5μM zinc pyrithione for 5 minutes alone (48.9 ± 12.3%) or immediately prior to TNFα (56.8 ± 9.1%) significantly decreased AP-1 binding activity to below control levels (Figures 10 and 11). This effect appears to be specific for zinc as treatment with sodium pyrithione or DMSO in conjunction with TNFα (125.8 ± 7.1% and 125.5 ± 4.0%, respectively) did not alter the control levels of AP-1 binding activity. No significant changes in Sp1 binding activity were found with any of the treatments (Figures 10 and 11). Thus, treatment of cells with zinc pyrithione alone or in conjunction with IR or TNFα significantly decreases AP-1 binding activity to below control levels.

At this point, it was of interest to briefly investigate the effect of zinc inhibition of TNFα-induced NFκB binding activity on apoptosis. The role of NFκB in apoptosis is controversial: there is evidence to suggest NFκB is involved in both the promotion of and the protection against apoptosis. Here, TNFα (20ng/ml) was found not to induce apoptosis (1.7 ± 0.2%, Figure 12), despite a 4.5-fold increase in NFκB binding activity. However, pretreatment with 5μM zinc pyrithione for five minutes (1.8 ± 0.4%), which

abolished NFκB binding activity, did not render the cell more susceptible to TNFα induced apoptosis (Figure 12).

Figure 10. Effect of TNFα and zinc pyrithione on transcription factor binding activity. The binding activity of nuclear extracts to ^{32}P-labelled concensus sequences for the transcription factors NFκB, AP-1 and Sp1 were analyzed by EMSA. The nuclear extracts were prepared following two hours of experimental treatment, and equal amounts of nuclear protein (5μg) were assayed for binding activity. NFκB binding activity: Cells treated with TNFα (20ng/ml) showed an increase in NFκB binding activity, which was abolished by treatment with 5μM zinc pyrithione (ZnPyr) for 5 minutes immediately prior to the incubation with TNFα. Treatment with sodium pyrithione (NaPyr) or DMSO prior to the incubation with TNFα did not affect the TNFα-induced increase in NFκB binding activity. Zinc pyrithione treatment alone had no effect on NFκB binding activity. AP-1 binding activity: The binding activity of AP-1 was not altered by TNFα. However, treatment with zinc pyrithione (5μM for 5 minutes) alone or prior to the incubation with TNFα decreased the basal levels of AP-1 binding activity. This effect was not seen by treatment with sodium pyrithione or DMSO prior to the incubation with TNFα. Sp1 binding activity: The binding activity of Sp1 was unaltered by all treatments. (figure is representative of at least 3 independent trials)

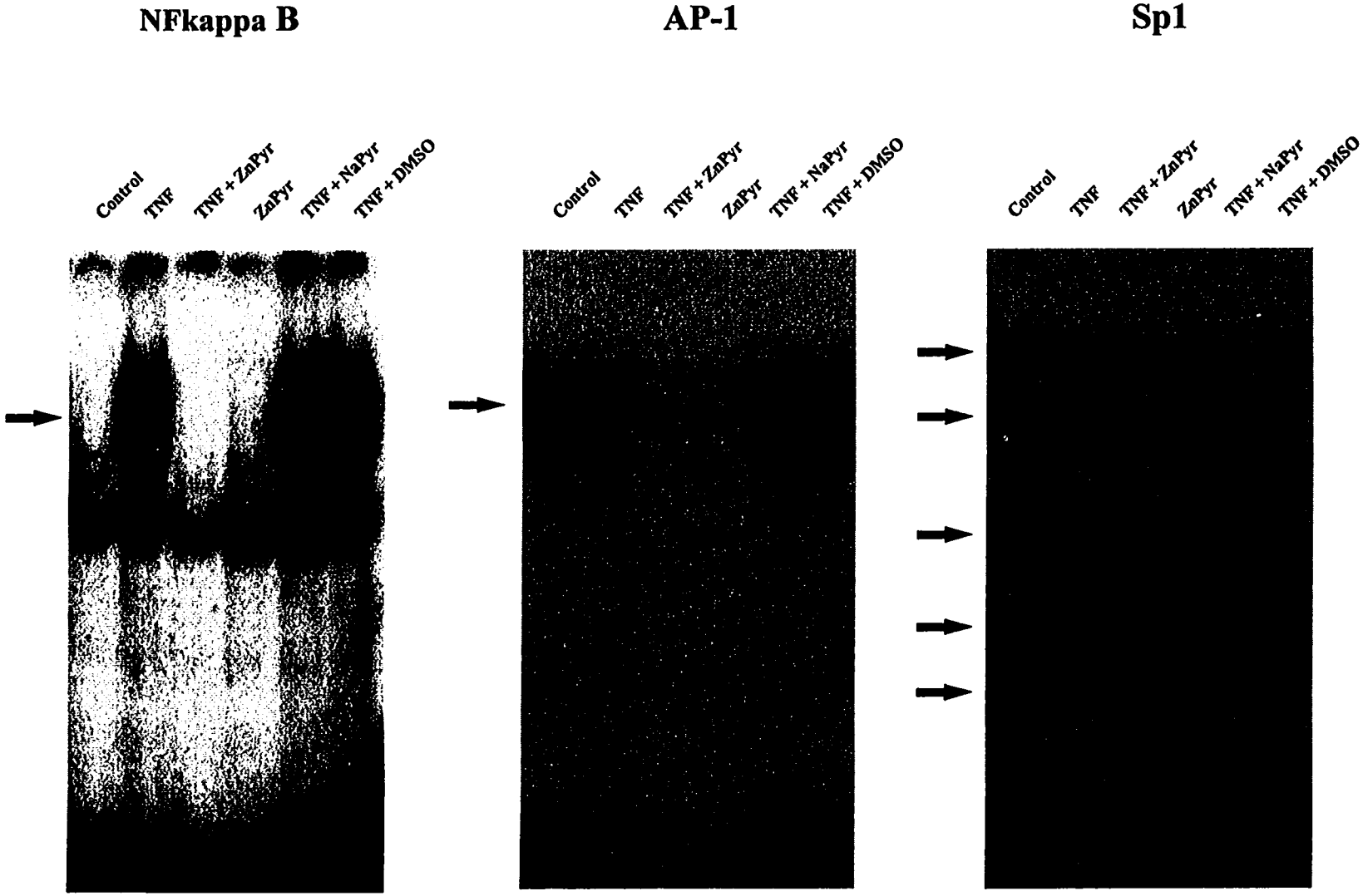

NFkappa B
AP-1
Sp1
Control
TNF
TNF + ZnPyr
ZnPyr
TNF + NaPyr
TNF + DMSO

Figure 11. Quantification of transcription factor activity in response to TNFα and zinc pyrithione. The transcription factor binding activity was assessed by EMSA following two hours of experimental treatment and was analyzed by quantitative densitometry. The values are expressed as a percentage of control levels. Cells treated with TNFα (20ng/ml) exhibited a 4.5-fold increase in NFκB binding activity. Treatment with 5μM zinc pyrithione (ZnPyr) for 5 minutes immediately prior to the incubation with TNFα significantly reduced nuclear NFκB binding activity to below control levels. Treatment with sodium pyrithione(NaPyr) or the carrier DMSO had no effect on the TNFα-induced increase in nuclear NFκB binding activity. Zinc pyrithione treatment alone had no effect on nuclear NFκB binding activity. The nuclear binding activity of the transcription factor AP-1 was not altered by incubation of the cells with TNFα. However, zinc pyrithione treatment alone or prior to the incubation with TNFα decreased AP-1 binding activity to below control levels. Treatment with sodium pyrithione or DMSO prior to the incubation with TNFα had no effect on AP-1 binding activity. No significant changes in Sp1 binding activity were found with any of the treatments. (*$P<0.05$ versus control group, °$P<0.05$ versus TNFα group, the n value for each treatment is as follows: control n=6, TNFα n=5, TNFα + ZnPyr n=4, ZnPyr n=3, TNFα + NaPyr n=3, TNFα + DMSO n=3)

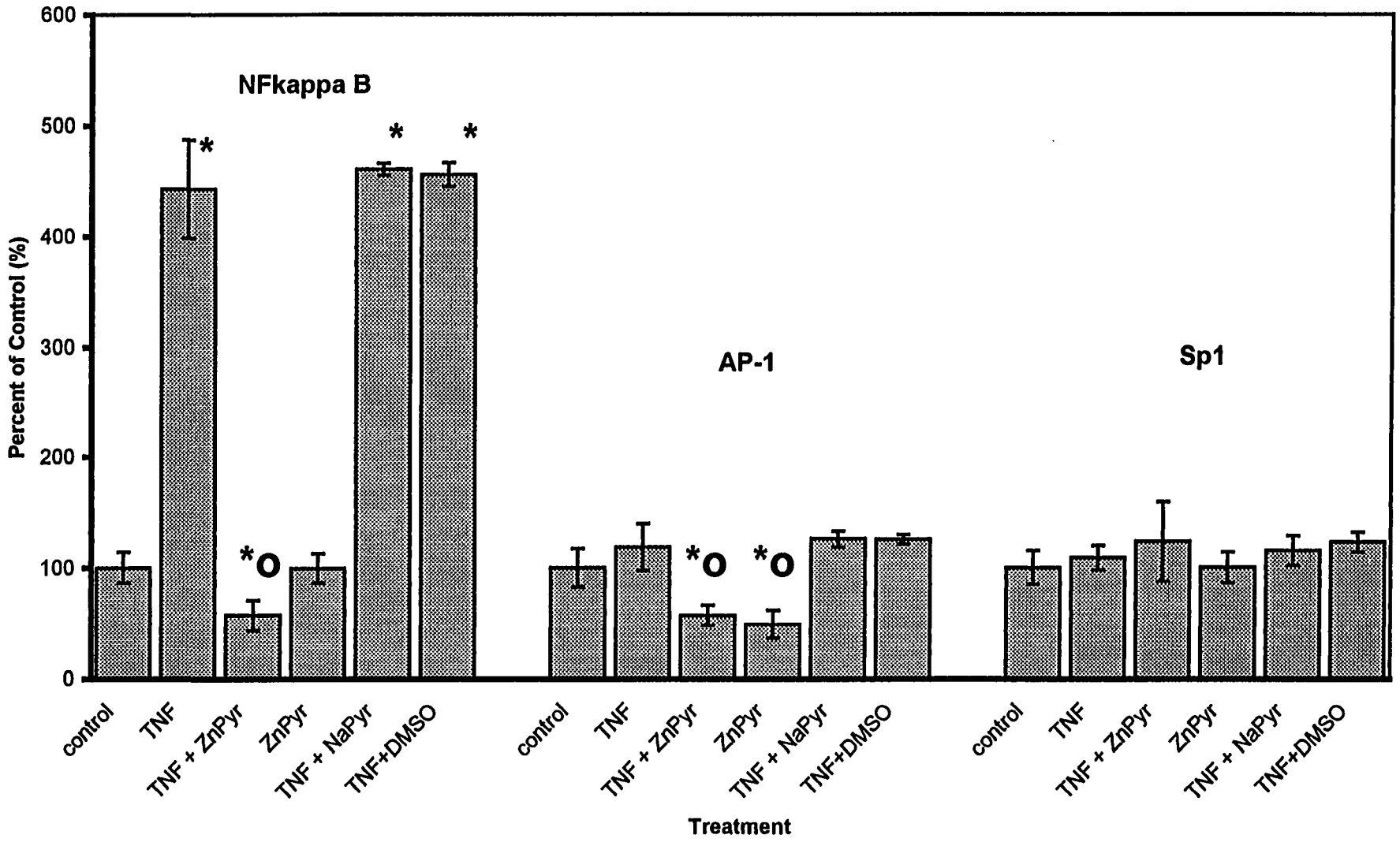
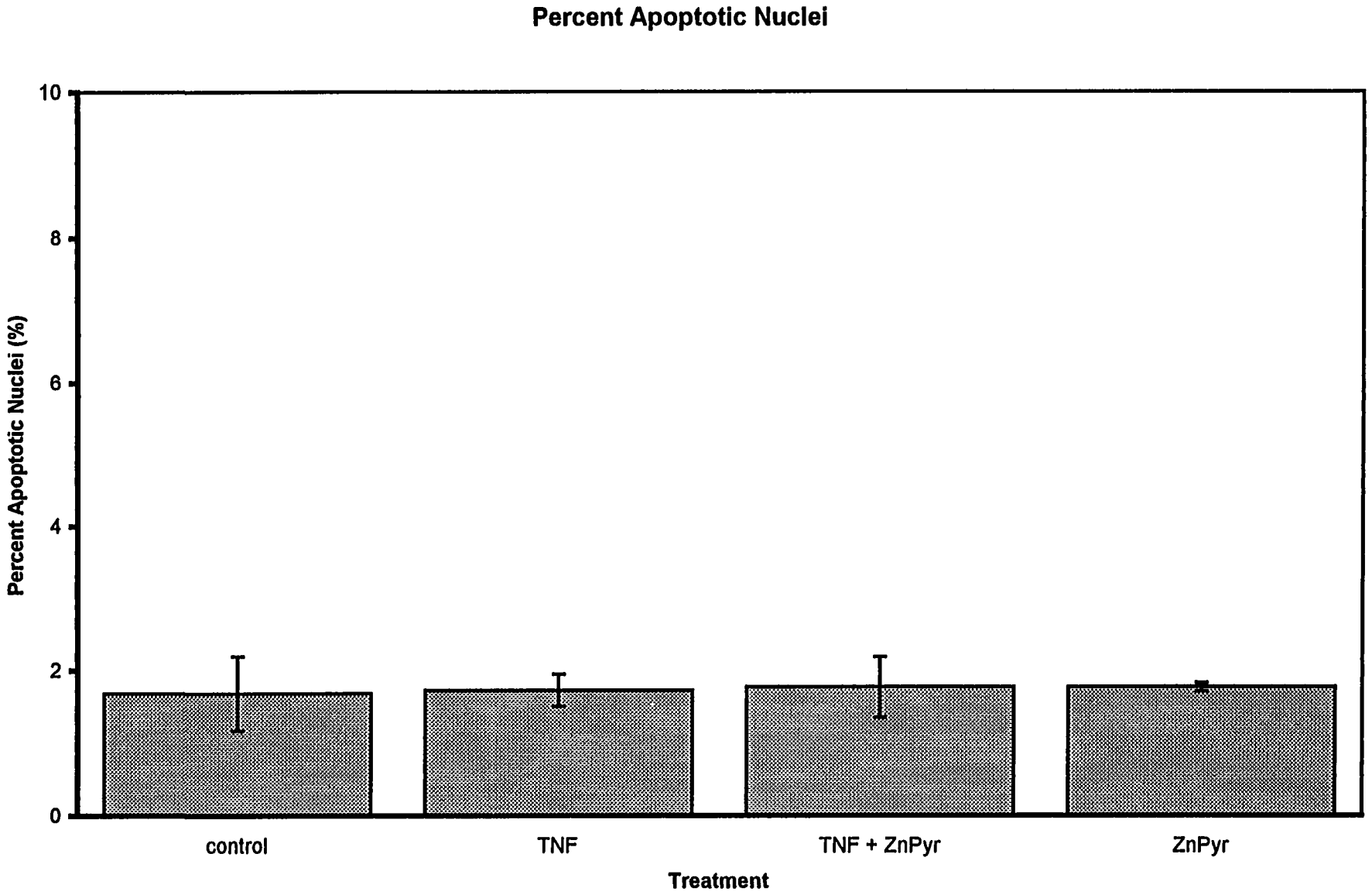

TNFalpha-Induced Transcription Factor Binding Activity: Effect of Zinc
NFkappa B
AP-1
Sp1
Percent of Control (%)
600
500
400
300
200
100
0
control
TNF
TNF + ZnPyr
ZnPyr
TNF + NaPyr
TNF+DMSO
Treatment

Figure 12. Effect of zinc on TNFα-induced apoptosis. The percent of cells with apoptotic morphology was assessed in Hoechst 33258-stained coverslips by determining the percent of nuclei displaying apoptotic morphology after 8 hours of treatment. The number of nuclei exhibiting apoptotic morphology from ten fields of view per coverslip were counted and expressed as a percent of the total number of nuclei. Incubation of the cells with TNFα (20ng/ml) did not induce significant apoptotic morphology above that observed in control cells. Treatment with 5μM zinc pyrithione (ZnPyr) for 5 minutes, alone, or immediately prior to incubation with TNFα, did not alter the occurrence of apoptotic nuclei. (n=4)


Percent Apoptotic Nuclei
Percent Apoptotic Nuclei (%)
10
8
6
4
2
0
control
TNF
TNF + ZnPyr
ZnPyr
Treatment

Effect of Zinc on IκBα

In view of the zinc-induced inhibition of both IR and TNFα-induced NFκB binding activity, we sought to determine the effect of zinc on IκBα, the cytosolic inhibitor of NFκB. Western blots of cytosolic extracts obtained two hours post-treatment were probed for the IκBα protein (Figure 13). In agreement with the observed increases in nuclear NFκB binding activity, both IR (1000 Rads) and TNFα (20ng/ml) treated cells showed significant decreases in cytosolic IκBα levels (84.0 ± 2.1 and 55.3 ± 6.5%, respectively), suggesting that degradation of IκBα led to the nuclear translocation and increased binding activity of NFκB (Figure 14). Treatment with zinc pyrithione (5μM for 5 minutes) immediately post-IR, or prior to TNFα treatment further decreased IκBα levels (63.0 ± 3.6 and 37.1 ± 6.8%, respectively). However, as shown above, this decrease in cytosolic IκBα was not associated with an increase in nuclear NFκB binding activity as zinc treatment was found to abolish the observed increase in nuclear NFκB binding activity seen with IR and TNFα (Figures 8 and 10). This effect appears to be specific for zinc as treatment with sodium pyrithione had no effect on IκBα levels induced by IR or TNFα alone (87.0 ± 4.4 and 56.1 ± 7.1%, respectfully, Figure 14). Furthermore, treatment of the cells with zinc pyrithione alone (57.8 ± 13.2%) significantly decreased cytosolic IκBα levels but did not increase nuclear NFκB binding activity above control.

Figure 13. Cytosolic levels of IκBα are decreased by γ-irradiation, TNFα and zinc. Cytosolic extracts were prepared from cells following two hours of experimental treatment. Equal amounts of cytosolic protein (3μg) were then probed for IκBα by western blotting. IκBα levels were decreased in cells exposed to IR (1000 Rads) and in TNFα (20ng/ml) treated cells. Cells treated with 5μM zinc pyrithione(ZnPyr) for 5 minutes immediately post-IR or prior to TNFα treatment showed a further decrease in IκBα levels. Treatment with sodium pyrithione (NaPyr) had no effect on IκBα levels induced by IR (see Figure 14) or TNFα. Zinc pyrithione treatment alone decreased cytosolic IκBα levels noticeably. (figure is representative of at least 3 independent trials)

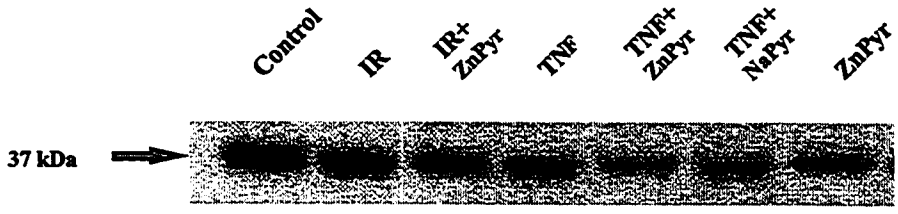
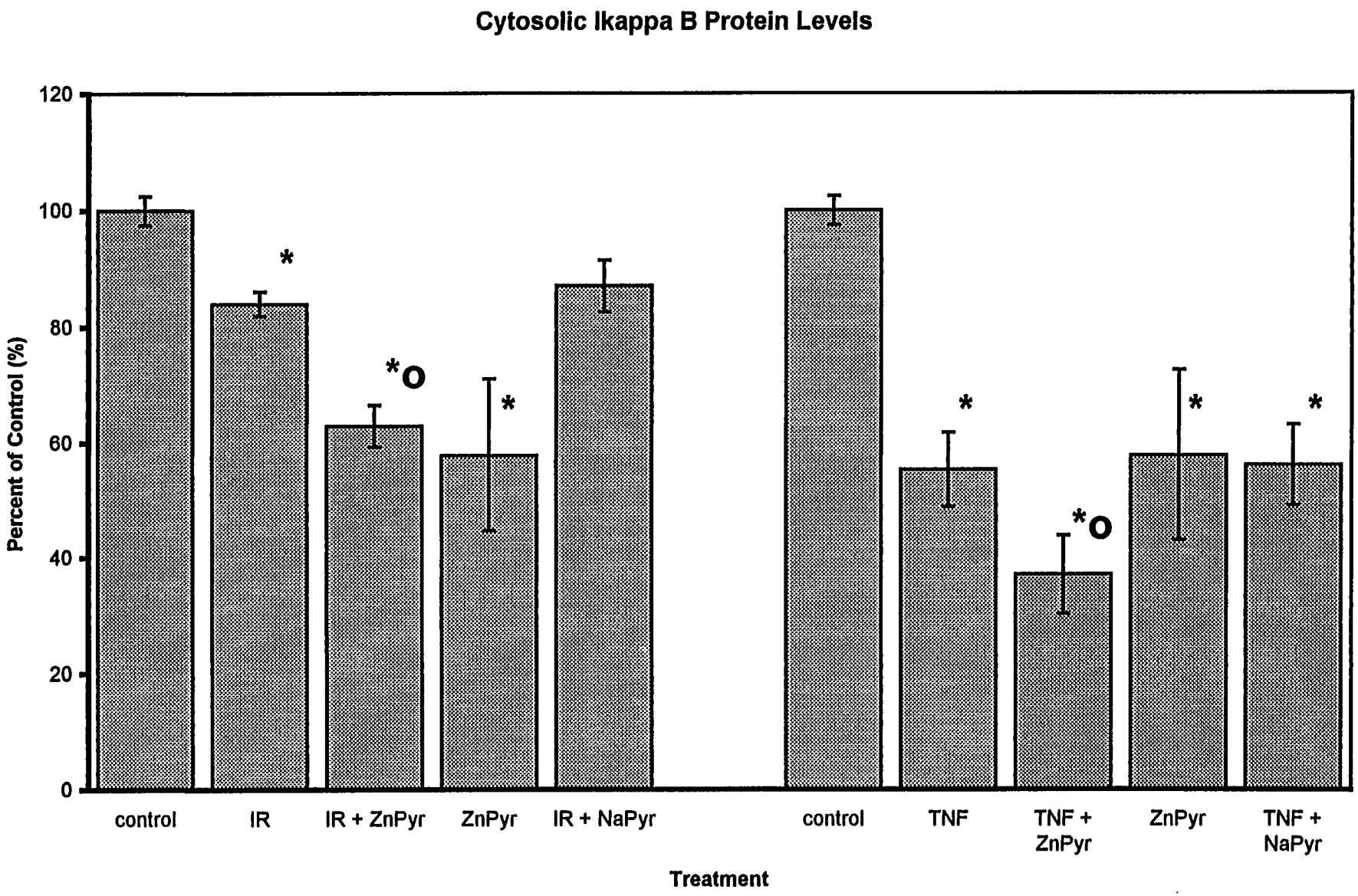

Control
IR
IR+ ZnPyr
TNF
TNF+ ZnPyr
TNF+ NaPyr
ZnPyr
37 kDa

Figure 14. Quantification of cytosolic IκB. Cytosolic levels of the protein IκB were probed by western blotting following two hours of treatment. The western blots were then analyzed by quantitative densitometry and cytosolic IκB levels were expressed as a percentage of control levels. IR (1000 Rads) and TNFα (20ng/ml) treated cells showed significant decreases in cytosolic IκB levels. Treatment with 5μM zinc pyrithione (ZnPyr) for 5 minutes immediately post-IR, or prior to TNFα treatment further decreased IκB levels. Treatment with sodium pyrithione (NaPyr) had no effect on IκB levels induced by IR or TNFα alone. Zinc pyrithione treatment alone significantly decreased cytosolic IκB levels. (*P<0.05 versus control, °P 0.05 versus IR, ˙P 0.05 versus TNFα, the n value for each treatment is as follows: control n=6, IR n=3, IR + ZnPyr n=3, ZnPyr n=3, IR + NaPyr n=3, TNFα n=6, TNFα + ZnPyr n=3, TNFα + NaPyr n=3)

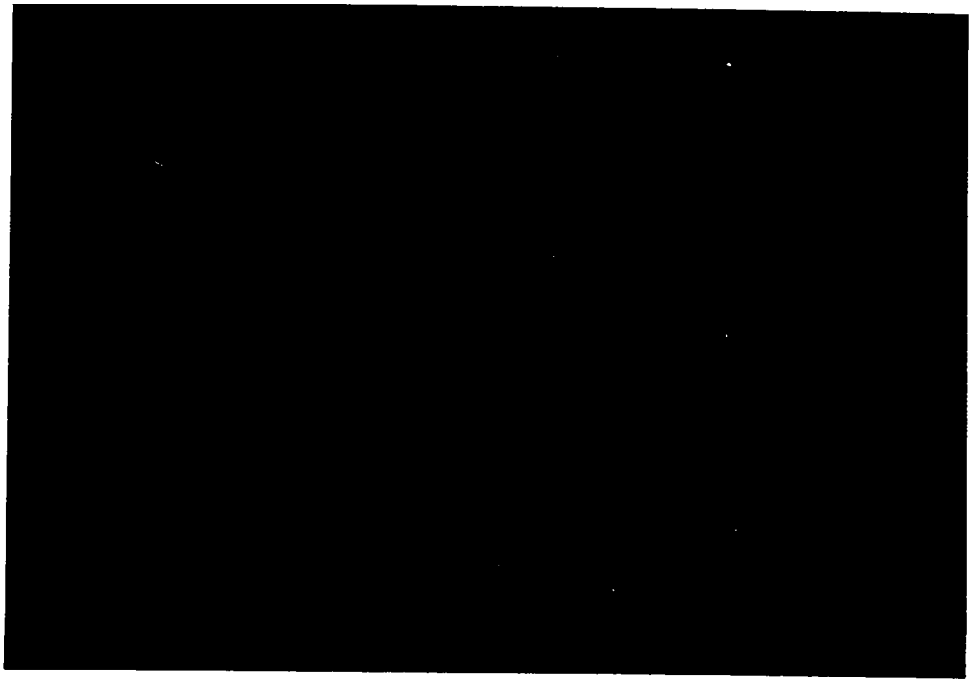

Cytosolic Ikappa B Protein Levels
Percent of Control (%)
120
100
80
60
40
20
0
*
*o
*
*
*
*o
*
*
control
IR
IR + ZnPyr
ZnPyr
IR + NaPyr
control
TNF
TNF + ZnPyr
ZnPyr
TNF + NaPyr
Treatment

Increased NFκB Binding Activity is Associated with Increased Apoptosis

Since TPEN-induced apoptosis was found not to be associated with an increase in NFκB binding activity, we attempted to manipulate the expression of NFκB binding activity in cells treated with TPEN to determine the effect, if any, on apoptosis. Co-incubation of HUVEC with TPEN (10μM) and TNFα (20ng/ml) resulted in an increase in apoptotic morphology (24.2 ± 2.0%, Figures 15 and 16) when compared with TPEN alone (13.9 ± 1.3%). TNFα alone (1.7 ± 0.2%) did not induce apoptosis. Co-incubation of HUVEC with TPEN and TNFα significantly increased the NFκB binding activity (401.0 ± 10.8%) in nuclear extracts compared to both control (100.0 ± 14.0%) and TPEN (110.8 ± 6.6%) treated cells (Figures 17 and 18), although not significantly differently from cells treated with TNFα (433.4 ± 44.4%) alone. Thus, co-incubation of TPEN with TNFα potentiated the TPEN-induced apoptosis, although it did not increase overall NFκB binding.

Figure 15. Morphological identification of apoptotic nuclei by Hoechst 33258. Cells were stained with Hoechst 33258 and examined for apoptotic nuclear morphology following 8 hours of experimental treatment. Panel A shows control cells, with little evidence of apoptotic morphology. Nuclei exhibiting apoptotic morphology were also absent in zinc pyrithione (5μM) and TNFα (20ng/ml, Panels J and D, respectively). Nuclei exhibiting apoptotic morphology are present in irradiated (1000 Rads), TPEN (10μM) and Act D (0.1μg/ml) treated cells (Panels B, E and G, respectively). Cells treated with zinc pyrithione immediately post-IR exhibited less apoptotic nuclei (Panel C) than cells receiving only IR, while cells incubated with CH (3μg/ml) immediately following IR (Panel H) also exhibited less apoptotic nuclei. CH alone did not induce significant apoptotic morphology (Panel I). Cells co-incubated with TPEN (10μM) and TNFα (Panel F) exhibited more morphologically apoptotic nuclei than cells receiving only TPEN (Panel E) or TNFα (Panel D). Zinc pyrithione treated cells were exposed to 5μM zinc pyrithione for five minutes alone, immediately post-IR. Nuclei representative of apoptotic cells are indicated by arrows. (figures are representative of at least 4 independent trials)

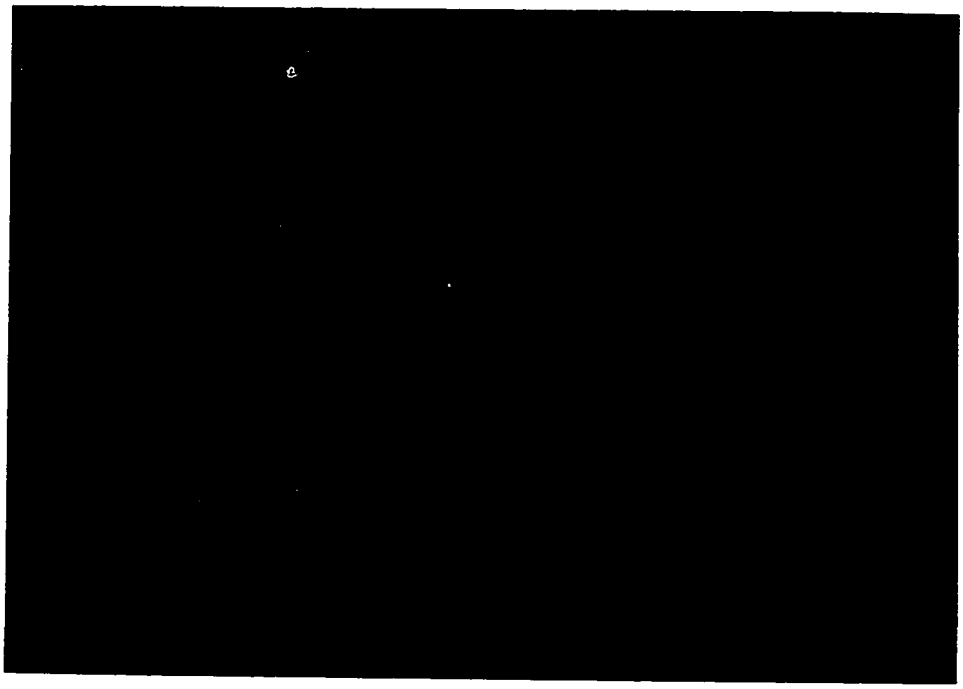
A



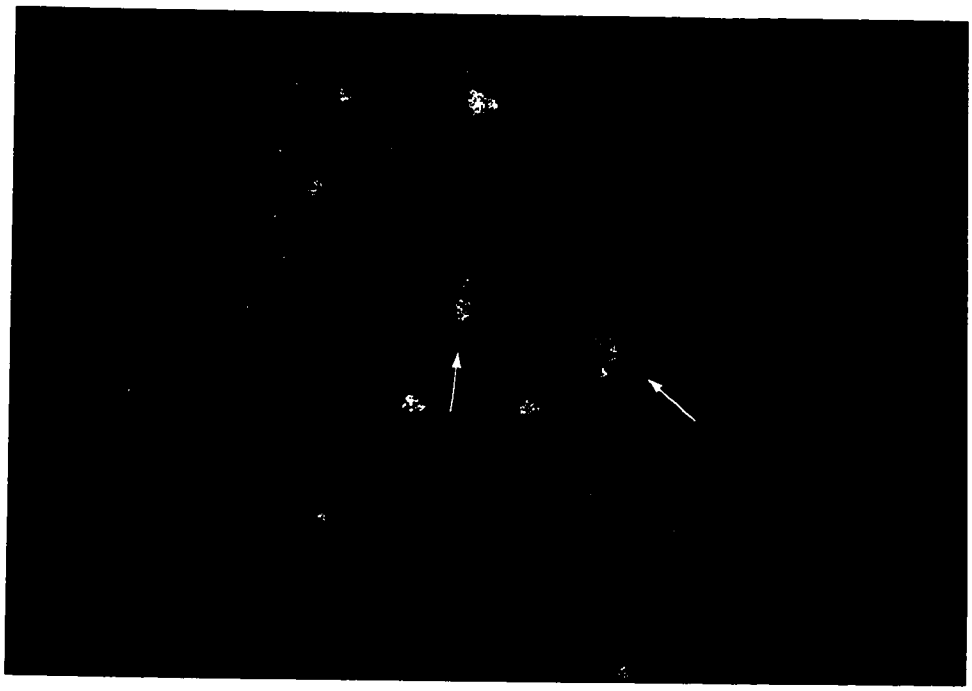
B

C

D

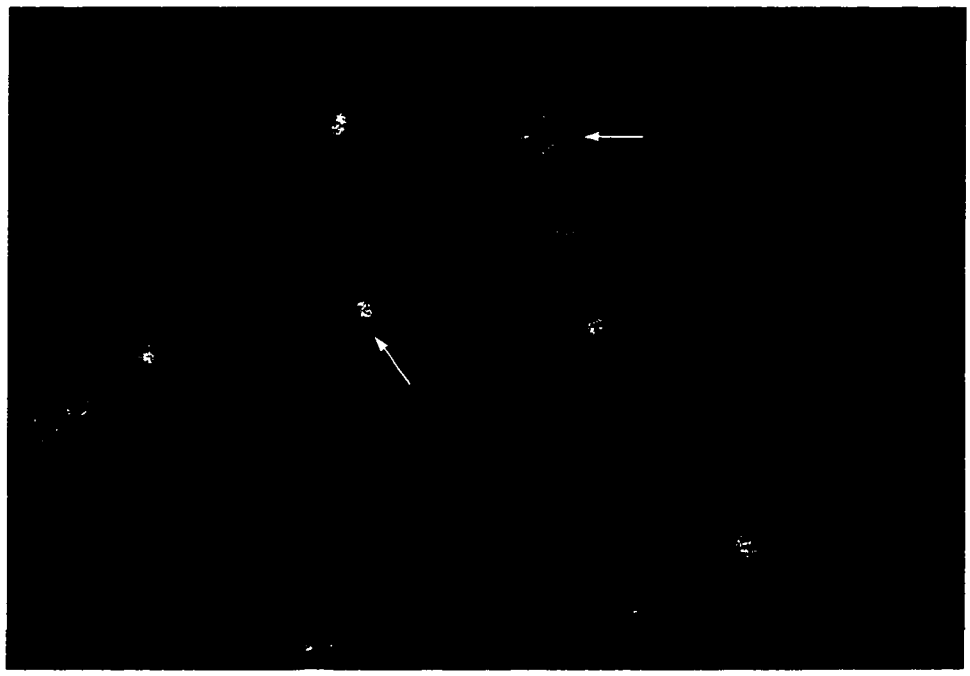


E

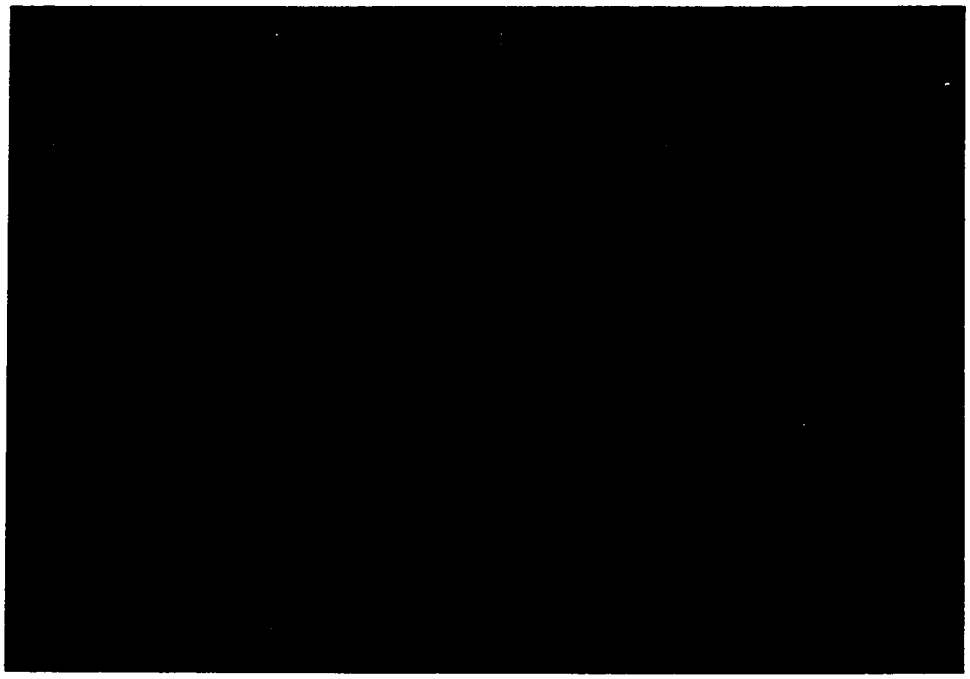


F

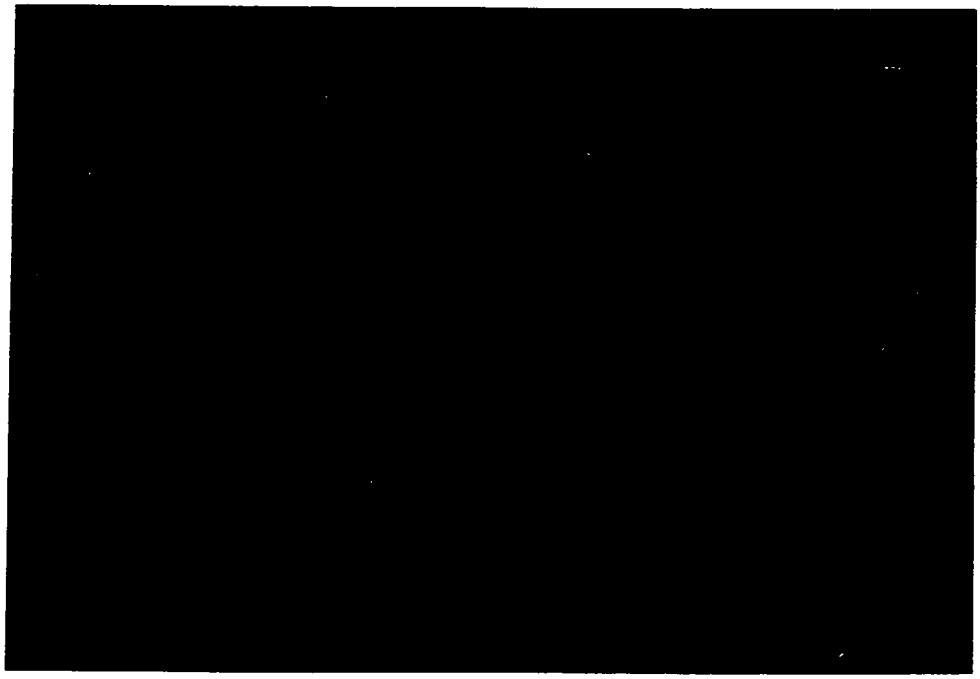
G



H



I



J

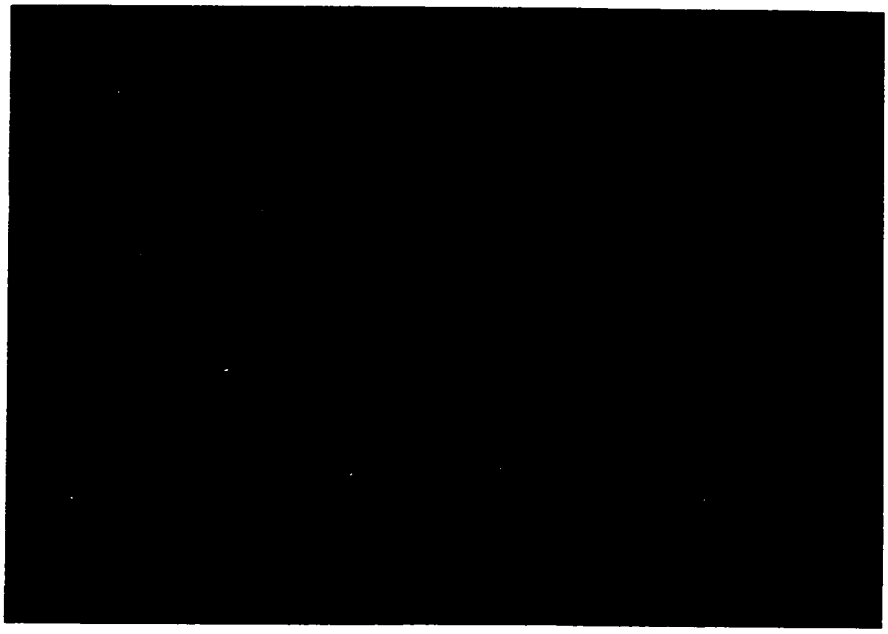


Figure 16. TNFα-induced potentiation of apoptosis. The percent of cells with apoptotic morphology was assessed in Hoechst 33258-stained coverslips by determining the percent of nuclei displaying apoptotic morphology after 8 hours of treatment. The number of nuclei exhibiting apoptotic morphology from ten fields of view per coverslip were counted and expressed as a percent of the total number of nuclei. Co-incubation of the cells with TNFα (20ng/ml) and TPEN (10μM) significantly increased the occurrence of apoptotic nuclei as compared to TPEN or TNFα treatment alone. The carrier, DMSO, had no effect on apoptotic cell death. (*$P<0.05$ versus control, °$P<0.05$ versus TPEN, n=4)

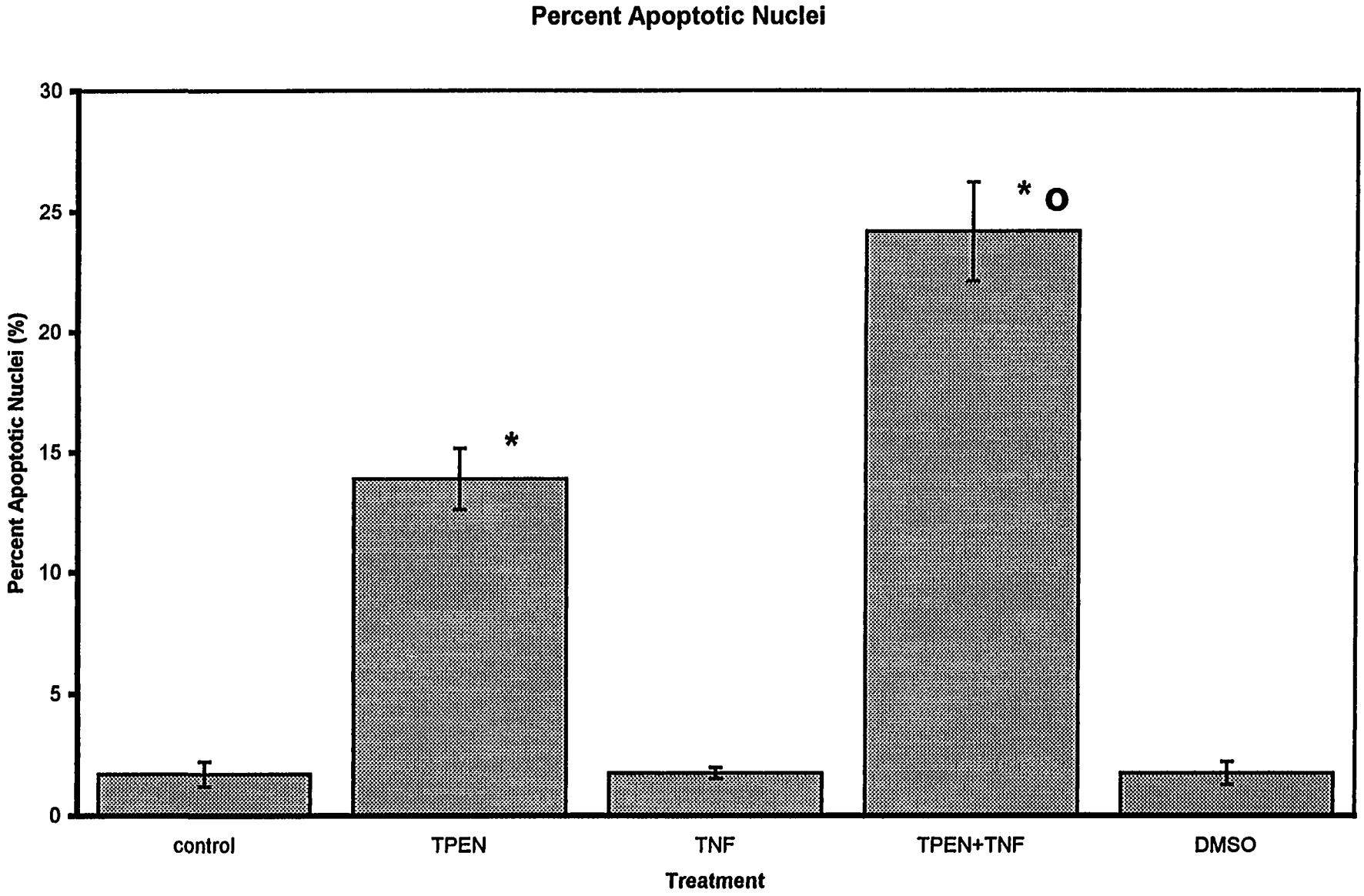

Percent Apoptotic Nuclei
Percent Apoptotic Nuclei (%)
30
25
20
15
10
5
0
*
* o
control
TPEN
TNF
TPEN+TNF
DMSO
Treatment

Figure 17. Effect of TPEN and TNFα on transcription factor binding activity. The binding activity of nuclear extracts to ^{32}P-labelled concensus sequences for the transcription factors NFκB, AP-1 and Sp1 was analyzed by EMSA. The nuclear extracts were prepared following two hours of experimental treatment, and equal amounts of nuclear protein (5μg) were assayed for binding activity. NFκB binding activity: Cells treated with TPEN (10μM) showed no change in NFκB binding activity, while nuclear extracts from cells treated with TNFα (20ng/ml) were dramatically increased in NFκB binding activity. A similar level of NFκB binding activity was expressed in cells co-incubated with TNFα and TPEN as was observed with cells treated with TNFα alone. AP-1 binding activity: The binding activity of AP-1 was unaffected by any of these treatments. Sp1 binding activity: The basal level of Sp1 binding activity was decreased by treatment with TPEN alone and by co-incubation with TPEN and TNFα. TNFα alone had no effect on Sp1 binding activity. (figure is representative of at least 3 independent trials)

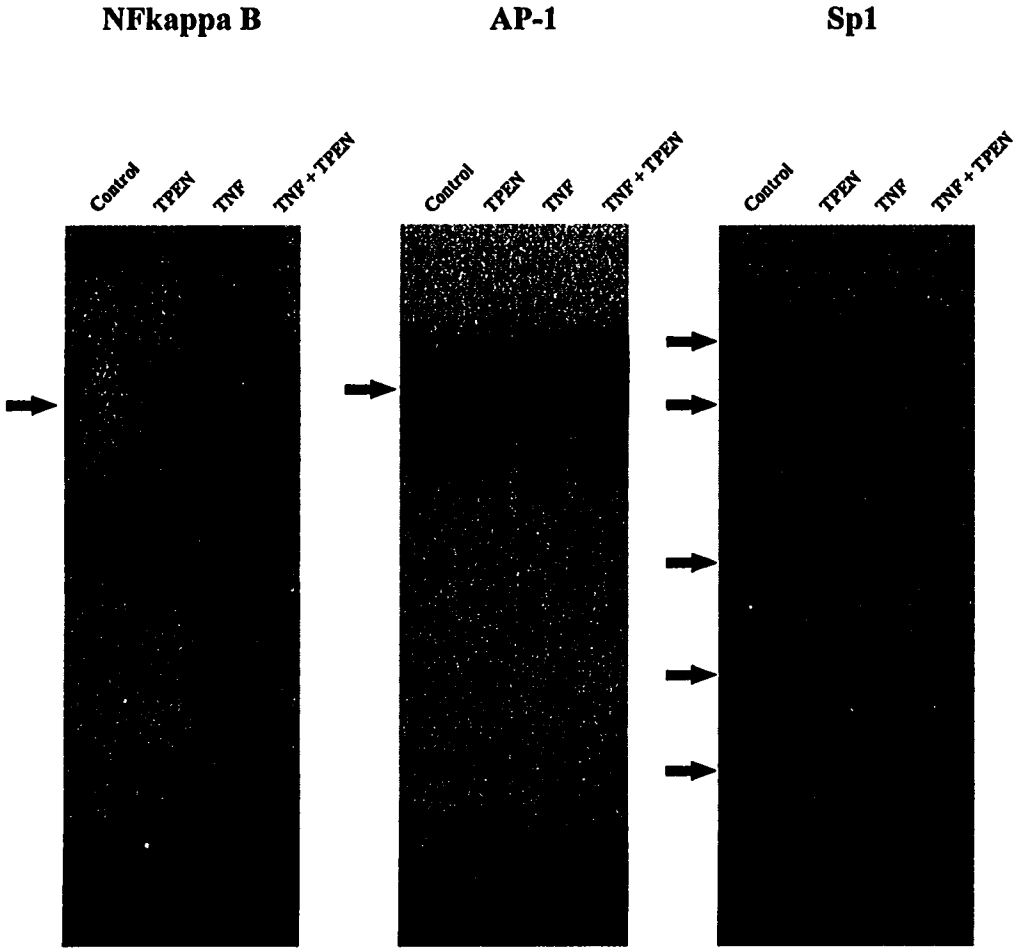
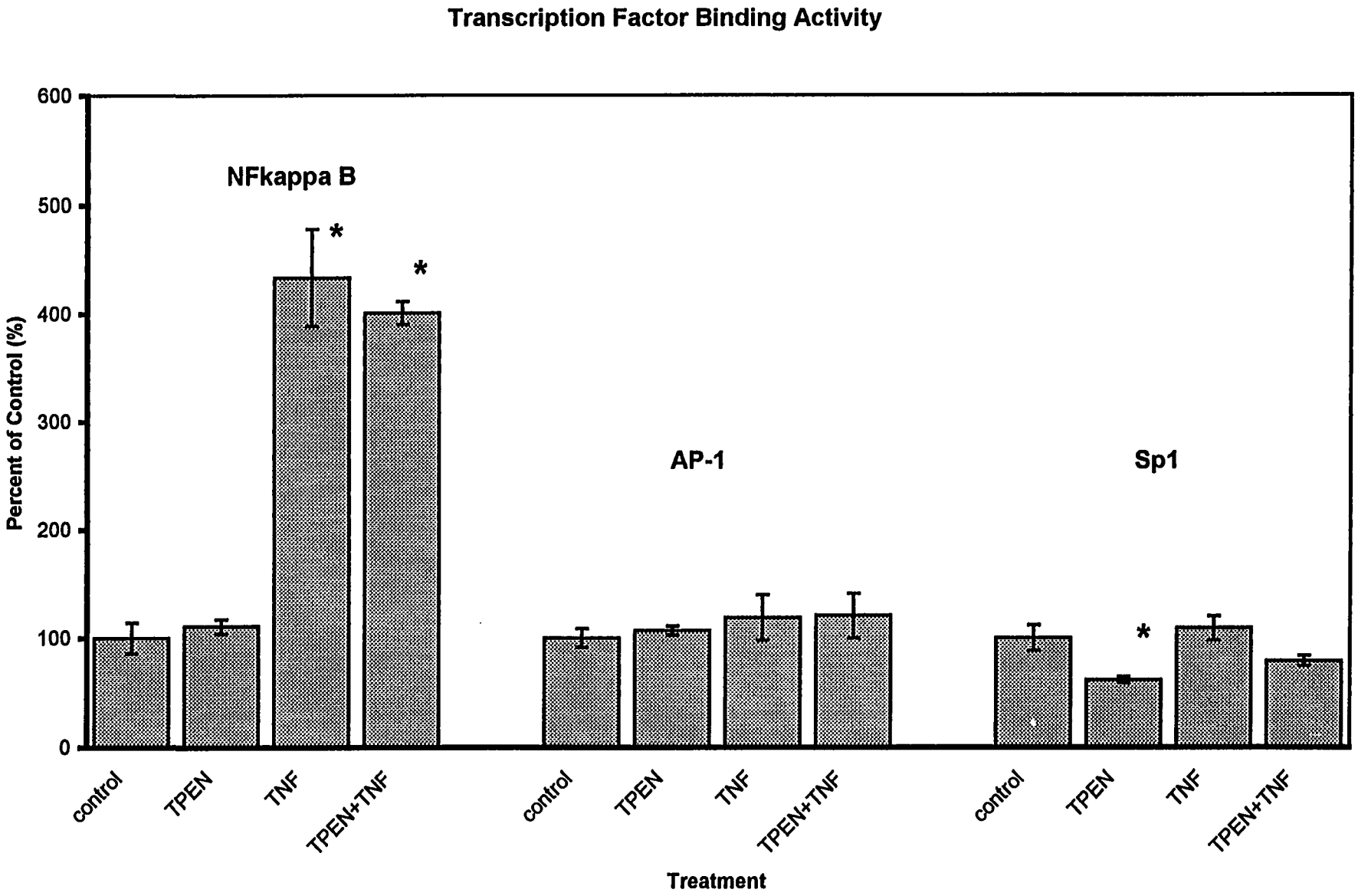

NFkappa B
AP-1
Sp1
Control
TPEN
TNF
TNF + TPEN
Control
TPEN
TNF
TNF + TPEN
Control
TPEN
TNF
TNF + TPEN

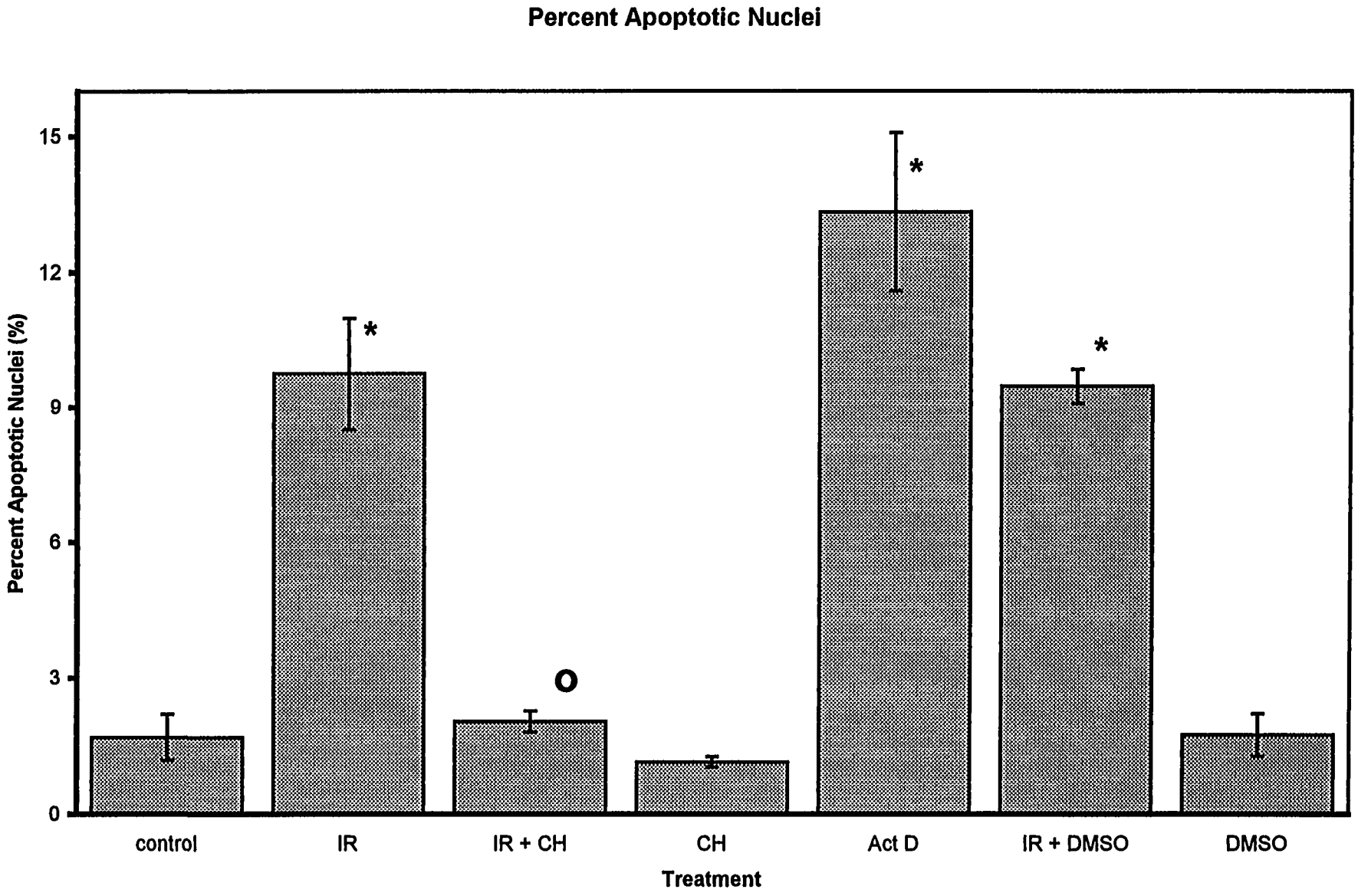
Figure 18. Quantification of transcription factor binding activity after co-incubation with TNFα. The transcription factor binding activity was assessed by EMSA following two hours of experimental treatment and was analyzed by quantitative densitometry. The values are expressed as a percentage of control levels. Nuclear extracts from cells treated with TPEN (10μM) showed no change in nuclear NFκB binding activity, while extracts from cells treated with TNFα (20ng/ml) increased binding activity significantly over control levels. Co-incubation of TPEN with TNFα did not significantly alter the level of NFκB binding activity seen with TNFα alone. No significant changes in AP-1 binding activity were seen with any of the treatments. Only TPEN induced a significant decrease in Sp1 binding activity, as compared to control values, while co-incubation of TPEN with TNFα did not achieve statistical significance as compared to control values. (*P <0.05 versus control group, the n value for each treatment is as follows: control n=6, TPEN n=3, TNFα n=5, TPEN+TNFα n=3)


Transcription Factor Binding Activity
600
500
400
300
200
100
0
Percent of Control (%)
NFkappa B
AP-1
Sp1
*
*
*
control
TPEN
TNF
TPEN+TNF
control
TPEN
TNF
TPEN+TNF
control
TPEN
TNF
TPEN+TNF
Treatment

Apoptosis and Inhibitors of Protein Synthesis

The requirement for protein synthesis in the induction of apoptosis was investigated (Figures 15 and 19). Incubation of HUVEC with the translational inhibitor CH (3μg/ml) immediately post-IR significantly reduced apoptosis (2.0 ± 0.2%) to levels seen in control cells (1.7 ± 0.5%) as indicated by morphological analysis (Figure 15) and quantitation of Hoechst 33258 stained nuclei (Figure 19). This effect was attributed to CH, as incubation of cells immediately post-IR with the carrier DMSO (9.5 ± 0.4%) had no effect on IR-induced apoptosis (9.7 ± 1.2%). CH alone (1.1± 0.1%) had no effect on basal levels of apoptosis. However, surprisingly, treatment of cells with the transcription inhibitor Act D (0.1μg/ml) significantly increased apoptosis (13.4 ± 1.7%).

Figure 19. Effect of inhibitors of transcription and translation on apoptosis. The percent of cells with apoptotic morphology was assessed in Hoechst 33258-stained coverslips by determining the percent of nuclei displaying apoptotic morphology after 8 hours of treatment. The number of nuclei exhibiting apoptotic morphology from ten fields of view per coverslip were counted and expressed as a percent of the total number of nuclei. Incubation of the cells with CH (3μg/ml) did not alter the occurrence of apoptosis as compared to control levels, while incubation with Act D (0.1μg/ml) significantly increased the occurrence of apoptotic nuclei. Incubation of the cells post-IR (1000 Rads) with CH significantly reduced apoptosis. Cells exposed to IR alone showed a significant increase in apoptotic morphology. Treatment of the cells post-IR with the carrier DMSO had no effect on the IR-induced apoptosis. DMSO treatment alone showed no significant difference in apoptotic morphology as compared to control levels. (*$P<0.05$ versus control, °$P<0.05$ versus IR, n=4)


Percent Apoptotic Nuclei
Percent Apoptotic Nuclei (%)
15
12
9
6
3
0
*
*
*
O
control
IR
IR + CH
CH
Act D
IR + DMSO
DMSO
Treatment

DISCUSSION

The ability of zinc to inhibit apoptosis has been well documented previously. However, while the anti-apoptotic properties of zinc have generally been attributed to the inhibition of the Ca^{2+}/Mg^{2+}-dependent endonuclease[49,105,109,329], recent results suggest that zinc may modulate apoptosis at several points upstream of the endonuclease[59,321,337]. In view of the ability of zinc to affect many cellular events, including transcription[319], we sought to determine if zinc might also function to prevent apoptosis by altering transcription factor binding activity. Therefore, in the present studies the ability of zinc to protect against apoptosis in HUVEC was explored and correlated with changes in transcription factor binding activity. Focus was placed on the transcription factors NFκB and AP-1 in view of their apparent involvement in the regulation of apoptosis.

Determination of Apoptosis

IR

Our evidence for IR-induced apoptosis complements previous studies[22,27-29,35]. IR is a well-established mediator of apoptosis in cultured cell systems. Numerous cell types have been reported to undergo apoptosis in response to a wide range of radiation doses[14,19,23,33,92,124]. In particular, both transformed and primary endothelial cells have been shown to induce an apoptotic response following exposure to IR[22,27-29,35,128]. Tissues rich in endothelial cells display increased sensitivity during radiation therapy[35], suggesting that the response of endothelial cells to IR warrants thorough investigation. Studies performed by Fuks and colleagues[27-29] have shown that a transformed line of bovine aortic endothelial cells undergoes substantial apoptosis in response to lower doses of IR than were used in this study. As the apoptotic response can vary markedly between transformed and primary cells and between cell types, and in particular, as endothelial cells can be significantly affected by matrix attachment and suspension, this

variability is not surprising.

Our study attempted to mimic the in vivo situation more accurately by using primary cell cultures and irradiating the cells as confluent monolayers in full medium, as outlined by Langley et al[35]. Langley et al. have shown that primary cultures of bovine adrenal capillary endothelial cells exposed to the same dose of IR as was employed here (1000Rads) resulted in apoptosis in approximately 20% of the population as determined by morphological analysis at 6 hours post-IR. These authors described IR-induced apoptosis as occurring in a discrete wave peaking at 6-10 hours post-treatment. In the present study, IR was also found to induce an apoptotic wave resulting in maximal apoptosis (approximately 10%) at 8 hours post-IR, and diminishing to control values by 24 hours (results not shown). Thus, using similar experimental conditions, we have obtained comparable results to Langley et al. in cultured HUVEC. In contrast, an earlier study by Eissner et al.[22] reported that 15% of the HUVEC population underwent apoptosis as measured at 24 hours post-IR with a dose of 500 Rads, again pointing out the inherent variability in this type of study. In general, our data fall within the range of observed radiation-induced apoptosis, and demonstrate that IR-induced apoptosis in HUVEC occurs in a wave, peaking at 8hrs and returning to control values by 24 hours.

TNFα

TNFα has been shown to mediate the induction of apoptosis in numerous cell types[63-65,127,161-163]. However, we did not observe a significant increase in apoptotic morphology in TNFα-treated HUVEC. Interestingly, consistent with recently emerging evidence suggesting that TNFα may induce both an anti- and a pro-apoptotic response within the cell[68], others have also shown that treatment with TNFα alone does not induce apoptosis[70,83,139,156,159,164]. Thus, vastly different responses to TNFα have been reported in the literature. Studies performed by several research groups have shown that both transformed and normal endothelial cells undergo apoptosis in response to TNFα[63,161-163], while others have shown that treatment

with TNFα alone does not affect cell viability[70,83,139]. Of particular interest is the response of HUVEC to TNFα. In our studies, exposure of HUVEC to TNFα for 8 hours did not result in significant apoptotic morphology. In addition, incubations of up to 24 hours also did not induce significant apoptosis as compared to control levels (results not shown). These results are consistent with others who have shown that TNFα is not capable of inducing apoptosis in either transformed or normal HUVEC[70,83,139]. In contrast, others have shown that treatment of HUVEC with TNFα induces apoptosis to a considerable extent[161-163]. The reasons for these discrepancies are not clear but may be attributable to the source and culture conditions of the cells. Alternatively, the ability of TNFα to induce apoptosis may vary depending on the source from which the TNFα was obtained. Here, the lack of a pro-apoptotic effect was not a result of a lack of biological activity of the TNFα, since treatment of a bovine aortic endothelial cell line with TNFα under the same conditions induced significant apoptosis (data not shown). Moreover, the NFκB activation data further show that the TNFα was biologically active. Thus, under the present experimental conditions, TNFα did not induce apoptosis in HUVEC.

Zinc

The chelation of intracellular zinc has been implicated in the induction of apoptosis in several cell types. Studies performed in the laboratory of Orrenius[102,103,165] have shown that treatment of rat and human thymocytes and Jurkat T cells with 10 μM TPEN, a potent heavy metal chelator, can induce significant apoptotic morphology and DNA laddering within four hours. Others[5,166] have also reported the induction of apoptosis following treatment of different cell types with TPEN. In the present study, incubation of HUVEC with 10 μM TPEN was found to induce significant apoptotic morphology compared to controls. In keeping with the apoptotic timeframe followed during the studies performed with IR and TNFα, TPEN-treated cells were incubated for 8 hours prior to staining for apoptotic morphology. While treatment of cells with TPEN does not mimic any known *in vivo* event[321], it does provide further support for a critical

role played by zinc in controlling cellular functions and provides a model for further insight into the mechanisms regulating the apoptotic pathway. The chelation of zinc may perturb cellular molecules requiring zinc for stability or catalytic activity, or may alter intracellular signaling pathways[320] in such a manner that apoptosis occurs[321]. As mentioned in Section 1.3.4, apoptosis induced by TPEN appears to proceed by a pathway requiring signal transduction between the cytoplasm and the nucleus[165]. Jiang et al. have suggested that zinc may stabilize the cytoplasmic membrane of cells, and that chelation of zinc by TPEN may alter membrane stability, resulting in the initiation of apoptotic signaling[165]. Here, we have presented a model system in which apoptosis is rapidly and significantly induced in HUVEC treated with TPEN.

Establishment of Transcription Factor Binding Activity

IR

Among numerous other effects, IR has been shown to induce both the activation of transcription factors[13,33,35,120-123] and apoptosis[14,19,22,23,27-29,33,92,124,128]. In the present study, IR was found to significantly increase the binding activity of the transcription factor NFκB in HUVEC, while the binding activities of AP-1 and Sp1 were not affected. The induction of NFκB by IR has been reported in several other cell systems and appears to vary depending on cell type[20,122,239-243]. Increases in NFκB binding activity have been reported from within minutes to hours following exposure of different cell types to wide ranges of IR doses (from 10 Rads to 50000 Rads). As the effect of IR on transcription factor activation in HUVEC had not been addressed prior to this study, this is the first report of a significant increase in NFκB following exposure of HUVEC to IR. The activation of NFκB was associated with a decrease in cytosolic IκB levels. The activated NFκB complex consisted of the p50 and p65 subunits, as determined by supershift analysis. In other cell types, both the expression of AP-1 subunits and the binding activity of AP-1 are increased following IR[290-292,338]. However, no significant increase in AP-1 binding activity was found following

treatment of HUVEC with IR. There have been no reports of an effect of IR on the binding activity of Sp1 in the literature.

TNFα

TNFα has been shown to be a potent inducer of NFκB in various cell types[64,65,164,221-225], including HUVEC[139,226]. Here, we confirm that TNFα induces the translocation of NFκB to the nucleus. Supershift analysis indicated that the NFκB binding activity in the nuclear extracts of TNFα-treated cells consisted of the p50 and p65 subunits, further supporting other reports that TNFα induces the activation and translocation of the 'classic' NFκB. The activation of NFκB was associated with a decrease in cytosolic IκB levels. TNFα has also been shown to induce the activation of AP-1 and Sp1 in human microvascular endothelial cells[164], while others have reported an increase in the gene expression of AP-1 subunits following treatment with TNFα[164]. In the present study, no significant increase was observed in the binding activity of either AP-1 or Sp1 following treatment with TNFα.

Zinc

The effect of TPEN on transcription factor binding activity has not yet been addressed in the literature. However, a recent report has suggested that TPEN-induced apoptosis requires the transduction of a signal originating in the cytoplasm to the nucleus[165], suggesting that the activation or suppression of transcription factors may be involved. Here, treatment of HUVEC with TPEN had no effect on the DNA binding activity of NFκB or AP-1 as compared to control cells. Interestingly, while zinc has been shown to be required for NFκB binding *in vitro*[267], the chelation of zinc with TPEN did not affect the basal level of NFκB binding activity. The basal level of Sp1 binding activity in the nuclear extracts of control cells was decreased following treatment of HUVEC with TPEN. This was not unexpected, as others have shown that thionein, a zinc chelator, will decrease Sp1 binding activity[318]. Presumably, the chelation of zinc can

interfere with the binding ability of Sp1 by disrupting the formation of zinc fingers.

Thus, consistent with observations made by others, nuclear NFκB binding activity was increased in response to both IR and TNFα. However, while others have reported increases in AP-1 and Sp1 binding activity in response to TNFα, or increases in AP-1 binding activity in response to IR, the present study was unable to confirm these results in HUVEC. The reasons for the discrepancies between the present results and published data may be attributable to cell-type specificity, the source or activity of the reagents or varying experimental protocol. Therefore, the present study reports that HUVEC responded to either IR or TNFα with the activation of the transcription factor NFκB, while the DNA binding activity of AP-1 and Sp1 remain unaffected by these treatments. Furthermore, treatment of HUVEC with TPEN did not alter NFκB or AP-1 binding activity, although TPEN did induce a significant decrease in the basal binding activity of Sp1.

This study sought to correlate the induction of apoptosis with changes in transcription factor binding activity. Of particular interest were the transcription factors NFκB and AP-1 in view of their potential involvement in the induction of apoptosis. Here we show that both IR and the heavy metal chelator TPEN induce apoptosis in HUVEC, while only IR induced a significant increase in NFκB binding activity. In turn, treatment of cells with TPEN inhibited the basal binding activity of Sp1. The activity of AP-1 was not altered by either treatment. Thus, IR-induced apoptosis may involve the activation of an NFκB-dependent pathway, while TPEN-induced apoptosis appears to occur independently of NFκB. Interestingly, TNFα did not induce apoptosis, although it did potently activate NFκB. The involvement of the transcription factor NFκB in the induction of apoptosis was further investigated by modulation of NFκB binding activity.

Zinc Inhibits Transcription Factor Binding Activity and Apoptosis

We sought to determine if protection against apoptosis could be associated with changes in

transcription factor binding activity. Numerous studies have shown that zinc can protect cells against the DNA fragmentation normally associated with apoptotic cell death, including IR-induced apoptosis[19,33]. More recently, zinc has been shown to exert a protective effect upstream of endonuclease[59,321,331]. In view of zinc's ability to interact with numerous proteins and the recent suggestion that zinc itself may function as a signaling molecule[320], we investigated the effect of zinc on apoptosis and transcription factor binding activity. HUVEC were treated immediately post-IR with zinc pyrithione, a zinc ionophore, which rapidly increases intracellular zinc levels. Zinc pyrithione protected against the IR-induced apoptosis and inhibited the increase in NFκB binding activity associated with IR. The basal binding activity of AP-1 was also inhibited by zinc pyrithione. Thus, inhibition of IR-induced apoptosis by zinc pyrithione is accompanied by the inhibition of NFκB and AP-1 binding activities. While zinc has been shown to inhibit apoptosis in a wide variety of systems, including IR-induced apoptosis in thymocytes[19,25,33], the use of a zinc-ionophore to suppress spontaneous and colchicine-induced apoptosis has also been reported[166]. Others have also reported an effect of zinc on NFκB binding activity[267,339,340]. *In vitro* studies have shown that while zinc is required for the DNA binding activity of NFκB, high concentrations of zinc (>1.5mM) will inhibit NFκB binding ability[267]. In addition, recent reports from Hennig's laboratory state that supplementation of cell medium with zinc inhibits both TNFα-induced activation of NFκB and the basal level of AP-1activity[339,340]. As both NFκB and AP-1 have been implicated in the promotion of apoptosis, inhibition of the binding activity of these factors may account for the protective effect of zinc pyrithione. Alternatively, in view of the apoptosis-suppressing qualities that have been attributed to NFκB, the inhibition of NFκB binding activity might have been expected to potentiate the IR-induced apoptosis. Indeed, inhibiting the activation of NFκB has been shown to induce or potentiate apoptosis induced by various agents, including IR[20,127,159]. As potentiation of IR-induced apoptosis was not observed despite the zinc pyrithione-induced inhibition of NFκB binding activity, the apoptosis-suppressing effect of zinc may be attributed to the inhibition of NFκB or to other apoptosis-suppressive qualities of zinc. It is possible that zinc might have inhibited apoptosis by

a mechanism other than the inhibition of transcription factor binding activity. As described earlier, zinc can affect protein interactions and may thus modulate the activity of enzymes involved in the transduction of apoptotic signaling. Zinc also contains antioxidant properties[322,323], which may contribute to the inhibition of IR-induced apoptosis by scavenging or preventing the generation of oxidative stress. Any of these effects, or others, may contribute to the inhibition of apoptosis induced by treatment with zinc pyrithione. Interestingly, Marchetti et al.[15] have shown that impeding disulfide bridge formation inhibits IR-induced apoptosis in thymocytes. In view of the ability of zinc to interact with sulhydryls, disulfide bridge formation may be blocked resulting in the inhibition of IR-induced apoptosis. The significance of the inhibition of basal AP-1 activity by zinc pyrithione is unclear. While apoptosis-promoting genes under the control of AP-1 have yet to be identified, the induction of apoptosis by IR may require a synergistic effect involving basal AP-1 activity[277,310]. Thus, IR-induced apoptosis may involve the activation of an NFκB dependent pathway, as zinc pyrithione inhibited both IR-induced apoptosis and NFκB activation.

Zinc Pyrithione also Inhibits TNFα-Induced NFκB

In view of the inhibition of IR-induced NFκB binding activity by zinc pyrithione, we sought to determine if zinc pyrithione could also inhibit NFκB induced by other means. Indeed, treatment of HUVEC with zinc pyrithione inhibited the TNFα-induced activation of NFκB. Thus, zinc appears to be acting at a point after the convergence of the TNFα- or IR-induced signal to activate NFκB, or alternatively, further downstream to inhibit the binding of activated NFκB. These results are supported by the recent observation from Hennig's laboratory that zinc supplementation inhibits TNFα-induced activation of NFκB[339,340]. We further investigated the effect of NFκB inhibition on apoptosis in TNFα-treated cells. Inhibition of NFκB binding activity by zinc pyrithione did not render HUVEC more susceptible to TNFα induced apoptosis. These results suggest that NFκB is not associated with a protective effect against TNFα-induced apoptosis, as the abolishment of NFκB binding activity by zinc did not

potentiate apoptotic cell death. However, others have shown that NFκB is associated with a protective effect against apoptosis[20,127,159]. Assuming NFκB is protective, there are several explanations as to why the inhibition of NFκB binding did not potentiate apoptosis under the present experimental conditions. Firstly, as discussed earlier, zinc may function elsewhere in the cell to protect against apoptosis[59,322,337]. Thus, while inhibition of NFκB binding activity may potentiate the cell to TNFα-induced apoptosis, other apoptosis-suppressing effects of zinc may prevent cell death from occurring. Secondly, HUVEC may not express the appropriate receptors required for the signaling of TNFα-induced apoptosis. While we did not investigate this possibility in the present study, Slowik et al have recently reported the expression of both TNFα receptors in this cell type[70]. Thirdly, the time frame for the assessment of apoptosis may have been insufficient. However, while the present study assessed apoptosis following 8 hours of experimental treatment, subsequent studies during which apoptosis was assessed for up to 24 hours did not reveal a potentiation to TNFα-induced apoptosis following inhibition of NFκB binding activity by zinc pyrithione. Nevertheless, zinc pyrithione was found to inhibit both IR- and TNFα-induced NFκB binding activity. We next sought to determine at what stage the inhibition of NFκB binding activity was occurring.

IκBα Disruption by Zinc Pyrithione

The effect of zinc pyrithione on IκB, the cytosolic inhibitor of NFκB, was investigated in order to determine the stage at which the inhibition of NFκB binding activity occurred. Two possible scenarios were envisioned: Firstly, zinc would inhibit the NFκB binding activity by disrupting the signal transduction pathways leading to the activation of NFκB. Thus, IR- or TNFα-induced degradation of IκB would be blocked. Secondly, zinc would inhibit the DNA binding ability of NFκB once translocated to the nucleus. Thus, IR- or TNFα-induced degradation of IκB would proceed and NFκB binding activity would be blocked at the DNA binding stage. However, treatment with zinc pyrithione alone was found to decrease cytosolic IκB levels and treatment with zinc pyrithione in combination with IR or TNFα further decreased

cytosolic IκB levels as compared with IR or TNFα alone. The effect of zinc pyrithione on IκB degradation is perplexing in view of the inhibition of NFκB binding ability by zinc pyrithione, as assessed by EMSA. Indeed, the increased NFκB binding activity in nuclear extracts from cells treated with either IR or TNFα corresponded to a decrease in IκB levels in cytosolic fractions. In view of the numerous effects of zinc within the cell, it is possible that the rapid rise in intracellular zinc following treatment with zinc pyrithione modulates signaling pathways resulting in the phosphorylation of IκB and its subsequent degradation[341]. While this could explain the decrease in cytosolic IκB levels, it does not account for the decrease in nuclear NFκB binding activity. As described earlier, NFκB contains a critical cysteine residue required for DNA binding activity[232,265] and in vitro studies have shown that NFκB binding activity is decreased by high concentrations of zinc[267]. The interaction of high concentrations of zinc with sulfhydryl groups may disrupt the critical cysteine residue required for NFκB-DNA binding ability and inhibit NFκB binding ability. Indeed, NFκB binding in vitro is inhibited by agents that modify free sulfhydryls[266]. It is possible that the effect of zinc on NFκB activation is two-fold: degradation of cytosolic IκB and inhibition of nuclear NFκB-DNA binding activity. Further investigations into the mechanisms regulating this effect are necessary. Also of interest was to speculate on the inhibition of AP-1 binding activity by zinc pyrithione.

Inhibition of AP-1 Basal Activity by Zinc Pyrithione

Treatment of HUVEC with zinc pyrithione was found to inhibit the basal DNA binding activity of AP-1. Others have shown that zinc will inhibit basal AP-1 binding activity [339,340], although a mechanism for this action has not been suggested. As AP-1 also contains a critical cysteine residue located within the DNA binding domain[278], it is possible that high concentrations of zinc may complex with this residue and inhibit DNA binding activity.

Increased NFκB is Associated with Increased Apoptosis

TPEN-induced apoptosis was not associated with an increase in NFκB binding activity. We sought to determine if increasing NFκB binding activity could potentiate the TPEN-induced apoptosis. In view of the potent NFκB-inducing potential of TNFα, and the apparent lack of effect of TNFα on apoptosis in HUVEC, we rationalized that incubation of HUVEC with TNFα would be an effective means of selectively activating NFκB without adding a complicating apoptosis component. Indeed, co-incubation of TPEN and TNFα did not affect the usual increase in NFκB binding activity normally observed with TNFα alone. However, this co-incubation produced a synergistic effect on apoptosis, with TNFα greatly potentiating the apoptosis observed with TPEN alone. These findings provide indirect evidence implicating the activation of NFκB in the induction of apoptosis. In view of these data it seems unlikely that NFκB has apoptosis-inhibiting activity in HUVEC. Since such apoptosis-suppressing qualities that have previously been attributed to NFκB[20,127,159], such activity in our studies would have been expected to inhibit the TPEN-induced apoptosis. Indeed, activation of NFκB by agents that do not induce apoptosis has been reported to protect cells against apoptosis[126,342,343]. As protection against TPEN-induced apoptosis was not observed by co-incubation with TNFα, a potent inducer of NFκB, it is possible that the chelation of zinc by TPEN may have disrupted a potentially protective effect of NFκB. In this scenario, the activation of NFκB by TNFα might have been associated with the expression of apoptosis-suppressing proteins, such as A20[70,159]. However, the chelation of zinc by co-incubation with TPEN might have disrupted the protective proteins, blocking the protective pathway induced by TNFα and further potentiating the cells to apoptosis by the apoptosis-inducing pathway initiated by TNFα. It is of relevance here that the apoptosis-suppressing protein A20 contains numerous zinc-binding domains[344]. Regardless, these results suggest that increasing NFκB binding activity may be associated with a potentiation to apoptotic cell death.

Apoptosis and Inhibitors of Protein Synthesis

Apoptosis is regulated by the relative expression of apoptosis-inducing and apoptosis-suppressing factors within the cell. Some of these factors may be expressed constitutively while others may be induced by apoptosis-modulating stimuli and it is the balance of these factors within the cell that determines if apoptosis will occur. The inhibition of IR-induced apoptosis by CH and Act D in cell types other than HUVEC has been reported[33]. Here, we confirm that IR-induced apoptosis can be inhibited by CH in HUVEC, suggesting the involvement of de novo protein synthesis in the apoptotic process. CH alone did not induce apoptosis. Thus, apoptosis induced by IR appears to require the synthesis of apoptosis-promoting factors. These results suggest that HUVEC require the de novo synthesis of apoptosis-promoting factors for apoptosis to occur. Interestingly, treatment of HUVEC with the transcription inhibitor Act D induced significant apoptosis, which in turn suggests that the continual gene expression of apoptosis-suppressing factors is required. The apparent contradictory effects of the translation inhibitor CH and the transcription inhibitor Act D are difficult to reconcile, but may result from differences in the effectiveness of these agents on the inhibition of protein synthesis, non-specific effects of the inhibitors, or by differences in mRNA and protein stability[192]. The degree to which protein synthesis is halted upon treatment with CH or Act D may depend on their respective concentrations, which in turn may influence the propensity of the cells to undergo apoptosis. As quantitation of protein synthesis inhibition was not determined in this study, differences in protein synthesis inhibition and apoptosis cannot be correlated. Furthermore, the effects of CH and Act D on the inhibition of protein synthesis are not specific. Indeed, Orrenius has reported that while different translation-inhibitors will inhibit protein synthesis to the same extent, they will not protect against apoptosis with the same efficiency[192]. These authors suggest that the protective effects seen with CH may be due in part to non-specific effects. In addition, the susceptibility of mRNA[345] encoding apoptosis-inducing or apoptosis-suppressing factors or of the factors themselves to degradation may account for the contradictory effects of CH and Act D on apoptosis. Here, a potential

scenario to explain the observed results is described: Inhibition of protein synthesis does not result in apoptosis, as apoptosis-suppressing factors are more stable than apoptosis-inducing factors. However, mRNA for apoptosis inducing factors is more stable, so when transcription is halted, protein synthesis for apoptosis-inducing factors continues, while therelative level of apoptosis-suppressing factors falls. The apparent contradictory effects of the protein synthesis inhibitors CH and Act D on the induction of apoptosis in HUVEC highlight the complexity of the mechanisms involved in the regulation of apoptosis.

CONCLUSION

The present study shows that zinc pyrithione inhibits IR-induced apoptosis in HUVEC and is associated with a decrease in induced NFκB binding activity. The basal binding activity of AP-1 is also inhibited by zinc pyrithione. As both NFκB and AP-1 have been implicated in the induction of apoptosis, these results suggest that zinc may function to prevent apoptosis by interfering with transcription factor binding activity. However, the mechanism by which zinc pyrithione acts to inhibit transcription factor binding activity was not resolved. Furthermore, increases in NFκB binding activity were also associated with a potentiation to apoptosis: Co-incubation of TPEN with TNFα, a potent inducer of NFκB, potentiated the apoptosis observed with TPEN alone. Thus, while these results suggest that the activation of NFκB contributes to the induction of apoptosis, both of which can be inhibited by zinc pyrithione, the ability of zinc to exert its protective effects by transcription factor modulation has not been resolved.

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